STUDIES ON THE 'IN VITRO' DEGRADATION OF BARLEY NITRATE REDUCTASE

Judith Finlayson

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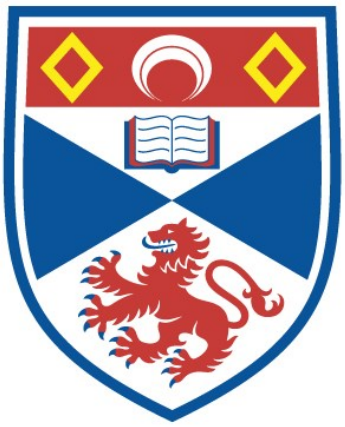
at the
University of St Andrews



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STUDIES ON THE *IN VITRO* DEGRADATION OF BARLEY NITRATE REDUCTASE

by

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A thesis submitted to the University of St. Andrews in application for the degree of Doctor of Philosophy.

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DECLARATION

I hereby declare that the following thesis is based on work carried out by myself, that the thesis is of my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr. J.L. Wray.

CERTIFICATE

I hereby declare that Judith Finlayson has spent nine terms in research work under my direction and that she has fulfilled the condition of Ordinance No.16 (St. Andrews), and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at Thames Polytechnic in October 1973 and graduated with the degree of Bachelor of Science (CNAA), First Class Honours in Applied Biology in November 1977.

In October 1977, I matriculated as a research student at the University of St. Andrews.

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SUMMARY

The effects of haem deficiency, as produced by treatment with laevulinic acid, on synthesis/assembly of NR in barley leaves was investigated. Interpretation of results was complicated by instability of NR *in vitro* therefore the age-dependent stability of NR was investigated.

The half-time of loss of NR activity in 4, 5 and 6 day old leaves of barley was found to be 358, 107 and 70 min respectively. BSA, PMSF, and 1,10-phenanthroline stabilised NR in extracts from 5 and 6 day old primary leaves, but BSA was most effective. The increased instability of NR with age of leaf correlated with increased conversion of the MW 203 000 NR complex to small NADH-CR species of MW 163 000, 61 000 and 40 000. The MW 163 000 CR species also possessed NADH-NR activity. BSA prevented and PMSF and 1,10-phenanthroline retarded the conversion of NR to the smaller CR species. The ability of BSA and the proteinase inhibitors to stabilise NR and inhibit conversion of NR to the small CR species indicates that the age-dependent *in vitro* stability of NR may be due to proteolytic degradation of NR. This suggestion is supported by the observation that trypsin cleaves purified NR into CR species sedimenting with the same coefficients as those observed in crude extracts. In addition, tryptic cleavage was retarded by the presence of BSA. Semi-purified preparations of maize root and barley leaf inactivating factors failed, however, to generate 3-4S CR species, the maize root inactivator appearing

to be active against the 3-4S CR species in addition to NR. It was concluded that neither factor was responsible for generating the small CR species observed in crude extracts.

The 40 000 MW CR species was purified and its MW confirmed using the method of Siegal and Monty (1966). The species was shown to possess NBT reductase activity but attempts to characterise the species with respect to haem content proved unsuccessful. Antibodies raised against the 40 000 MW CR species were found to inhibit all partial activities of NR and it was therefore concluded that this species, at least, was likely to be derived from NR.

Antibodies raised against purified barley NR were found to inhibit all NR activities to a similar extent. Pre-immune serum was found to stimulate NR activity and this was found not to be due to the presence of serum albumin. A protection of inhibition assay was developed for estimating NR-CRM. Negligible NR-CRM was detected in nitrate-less leaf extracts while substantial NR-CRM was found in ammonium-grown plant extracts. Pre-treatment of purified NR with NADH, but not with nitrate, was shown to preserve the enzyme, to some extent, from antibody inhibition.

In the General Discussion, evidence available regarding the *in vitro* breakdown of NR is reviewed and a model for the structure of higher plant NR is presented. In addition the probable route of genetic evolution of NRs is discussed. Evidence for the role of proteolysis in regulation of NR activity *in vivo* is also analysed.

ABBREVIATIONS

BSA	Bovine serum albumin
BVH	Reduced benzyl viologen
CR	Cytochrome *c* reductase
CRM	Cross-reacting material
cyt	Cytochrome
DCPIP	2,6-dichlorophenol-indophenol
$FMNH_2$	Flavin mononucleotide (reduced)
Leupeptin	Acetyl-L-leucyl-L-leucyl-L-arginal
Methyl viologen	1,1'-dimethyl-4,4'-bipyridium dichloride
MVH	Reduced methyl viologen
MW	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NBT, Nitroblue tetrazolium	2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride
NR	Nitrate reductase
PEG	Polyethylene glycol
pCMB	p-chloromercuribenzoate
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulphate
Szechrome NAS	Diphenylamine sulphonic acid Chromogene
TEMED	N,N,N',N'-tetramethyl-ethylene
TMBZ	3,3',5,5'-tetramethyl benzidine

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INTRODUCTION

NITRATE ASSIMILATION IN PLANTS

The assimilatory reduction of nitrate by plants is a fundamental biological process in which inorganic nitrogen is reduced to ammonium, which in turn combines with carbon skeletons to form the different biological nitrogenous compounds.

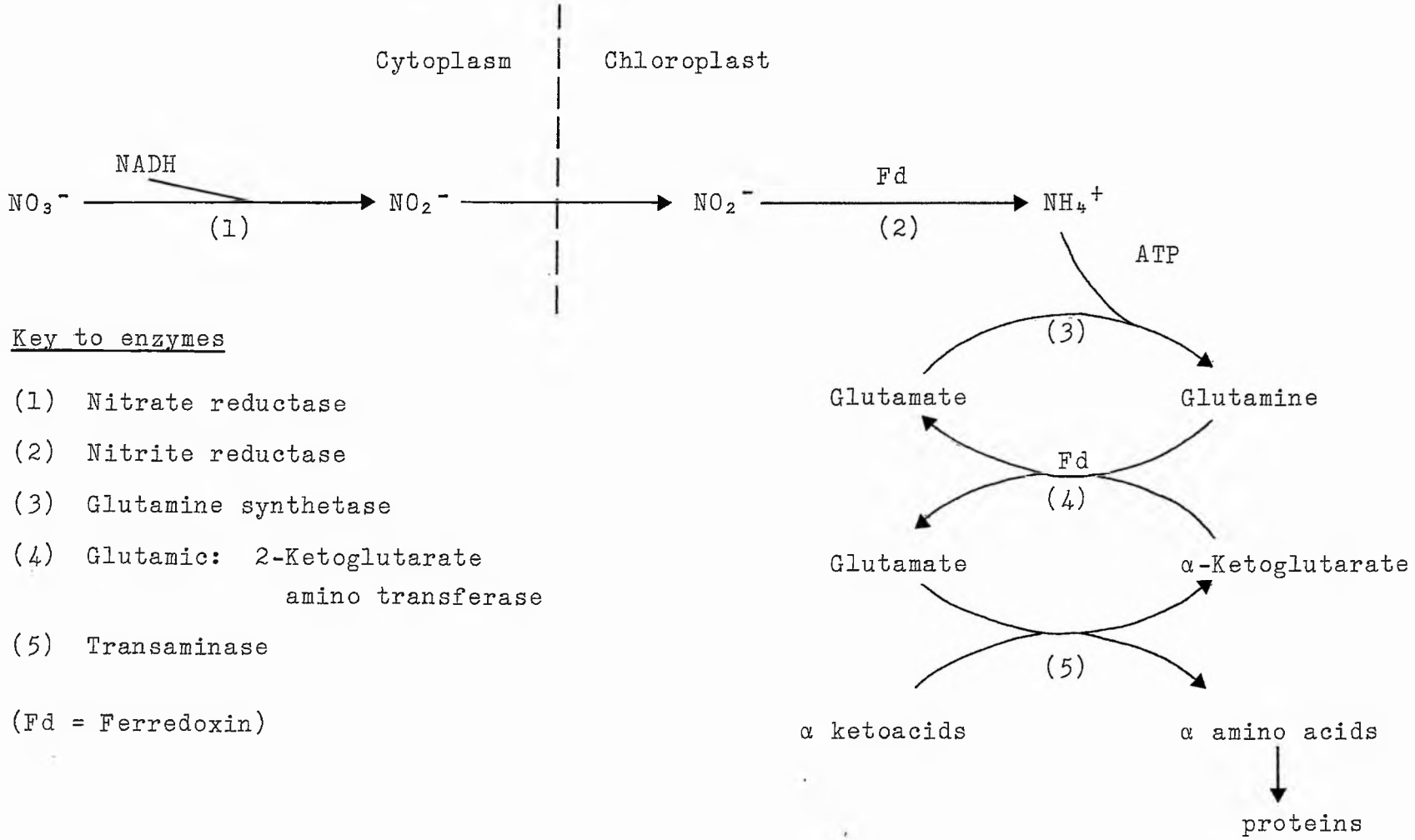
The majority of plant nitrogen is derived from mineralised nitrogen taken up from the soil, of which nitrate is the predominant form available to most cultivated plants. Nitrate assimilation is also of importance to legume species as nodule fixation may be greatly depressed by mineralised nitrogen, indeed soybean may obtain approximately 70% of its total nitrogen in mineralised form under field conditions (Harper and Hageman, 1972). Even though ammoniacal fertilisers are used almost exclusively, the ammonium is rapidly oxidised to nitrate by soil organisms. Nonetheless, most plants have the capability of absorbing and assimilating both nitrate and ammonium ions (Haynes and Goh, 1978) but nitrate is generally regarded as the safer form since ammonium is toxic and cannot accumulate without causing damage to the plant. Nitrate uptake is an energy-dependent process that proceeds more rapidly in strongly illuminated plants. High concentrations of soil nitrate also lead to increased nitrate uptake, nitrate ions probably activating a specific permease of root cells (Jackson *et al*, 1973).

NR catalyses the reduction of nitrate to nitrite, representing the first step in the incorporation of nitrate nitrogen into plant protein (Fig.1). Nitrate can either be reduced in the root, or transferred to the xylem for transport to the aerial parts of the plant for reduction there. Nitrate may also be stored in the leaf vacuole for later use when it is released into the NR-accessible, or metabolic pool (see Oaks, 1979). NR is present in most parts of higher plants but it is predominant in regions of active growth such as young leaves and root tips (Beevers and Hageman, 1969). When nitrate availability is low a greater proportion of nitrate is reduced by the roots, especially after storage pools of nitrate in the plant have been exhausted. There also appears to be intrinsic variability of tissue NR levels between species (Pate, 1973; Radin, 1977).

The intracellular location of NR in green tissues of higher plants is a controversial issue. Cell fractionation studies using marker enzymes indicate that the enzyme is soluble and located in the cytoplasm (Beevers and Hageman, 1972; Dalling and Tolbert, 1972) but Ritenour *et al* (1967) did not discount the possibility that NR may have a loose association with the outer chloroplast membrane which is lost during even mild extraction procedures. A chloroplast association for NR *in vivo*, however labile, would seem logical since this would avoid transport of the relatively toxic product of nitrate reduction through the cell for further assimilation by nitrite reductase which is located in the chloroplast. Recently

Fig.1

Pathway of Nitrogen Assimilation in Higher Plants



Key to enzymes

(1) Nitrate reductase

(2) Nitrite reductase

(3) Glutamine synthetase

(4) Glutamic: 2-Ketoglutarate amino transferase

(5) Transaminase

(Fd = Ferredoxin)

NR has been located in mycelial cells of *N. crassa* by immunohistochemical labelling with ferritin and the enzyme was found in the cell wall - plasmalemma region and in the tonoplast membranes (Roldán *et al*, 1982).

Since the synthesis of NR is inhibited by cycloheximide but not chloramphenicol it has been suggested that NR is synthesised on cytoplasmic 80S ribosomes (Schrader *et al*, 1967). More recently, it has been shown that all *nia* phenotypes of *N. tabacum* cell line mutants arise from mutations in nuclear genes (see Muller, 1982).

Nitrite formed by the reduction of nitrate enters the chloroplast where it is further reduced to ammonium by nitrite reductase, an enzyme which uses photosynthetically reduced ferrodoxin as cofactor (Vega and Kamin, 1977). Ammonium is further metabolised within the chloroplast by the glutamine synthetase-glutamate synthase (GOGAT) pathway (Miflin and Lea, 1976; 1977) illustrated in Fig.1. The glutamate formed in these reactions can then be used in the synthesis of the protein amino acids as well as for the synthesis of purines and pyrimidines. An alternative route of ammonium assimilation is via the reductive amination of α-ketoglutarate catalysed by glutamate dehydrogenase. However, Miflin and Lea (1976) consider that this route does not play a major role in nitrogen assimilation in higher plants except in circumstances of ammonium excess.

REGULATION OF NITRATE REDUCTION

The control of the overall process of nitrate assimilation is likely to be brought about, in part, by the regulation of the activities of the enzymes of the nitrate reducing pathway. Since the level of nitrite reductase is usually much higher than that of NR, nitrite accumulation is rarely observed. The reduction of nitrate to nitrite, rather than the further reduction of nitrite to ammonium, is therefore more likely to be the rate limiting step in nitrate reduction. Regulation of NR is generally considered to be the logical point to effect regulation of the inflow of nitrate-nitrogen into the plants' metabolism (Beevers and Hageman, 1969).

The induction of NR by nitrate in higher plants is well documented (Beevers and Hageman, 1969) and an obligatory requirement for nitrate as the specific inducer of NR has often been claimed. However, it has been shown that considerable enzyme levels are sometimes found in the absence of nitrate in tobacco cells (Muller and Grafe, 1978), cotton seedlings (Radin, 1974) and barley roots (Smith and Thompson, 1971). There is evidence that NR can be 'induced' in the absence of nitrate by a wide variety of compounds including cytokinins (Parkash, 1972), growth retardants (Knypl, 1974) and nitrite (Ingle *et al*, 1966; Kaplan *et al*, 1978).

Conflicting observations on the effect of ammonium on nitrate assimilation in higher plant systems have been

reported. Induction of NR in barley roots (Smith and Thompson, 1971), cotton roots but not shoots (Radin, 1975) and *Lemna minor* (Orebamjo and Stewart, 1975a) was inhibited by ammonium. However, nitrate induced appearance of NR was not influenced by ammonium in other tissues from higher plants (Beevers *et al*, 1965; Oaks *et al*, 1977). It has been suggested (Stravistava, 1980) that the wide range of response to ammonium may reflect the difference in regulation of NR in different tissues or it may be attributed, in some cases, to the composition of the media or methodological approach.

NR induction can also be inhibited by amino acids (Filner, 1966; Stewart, 1972; Radin, 1975) and amides (Stewart, 1972; Oaks, 1974; Radin, 1975) but the extent to which these compounds, especially amino acids, can be regarded as end product repressors of nitrate assimilation is considered debatable (Stewart and Rhodes, 1977). However, 'end product repression' can be readily demonstrated in cell cultures, roots and morphologically simple plants prompting Stewart and Rhodes (1977) to propose that these systems may be less 'buffered' and may accumulate potential repressors at the site at which control over NR is exerted.

A characteristic feature of higher plant nitrate metabolism is its susceptibility to a range of environmental conditions and, in most cases, this appears to be mediated by regulation of NR.

It has often been observed that light is essential for 'substrate' induction of NR (Hageman and Flesher, 1960; Travis *et al*, 1970a; 1970b) and that in light grown seedlings when transferred to the dark the enzyme activity decreases (Travis *et al*, 1970b; Upcroft and Done, 1972). However, as non-photosynthetic tissues such as roots are known to accumulate nitrate in the dark (Beevers and Hageman, 1969) and induction of NR in young maize seedlings has been demonstrated (Beevers *et al*, 1965; Travis and Key, 1971) it would appear that light does not have a direct role in assimilation of nitrate.

The intensity of light has been shown to influence NR activity (Fellipe *et al*, 1975) and it has been observed that nitrate accumulates in plants grown in shaded conditions (Hageman and Flesher, 1960). In addition, diurnal variation in NR activity is a commonly observed phenomenon (Duke *et al*, 1978; Upcroft and Done, 1976). It appears that oscillations in peak activity vary according to the prevailing conditions throughout the cycle and the number of peaks appears to be species-dependent.

Rapid changes in the activity of NR in response to other environmental factors such as temperature, pH, CO_2 and O_2 tensions, water potential and mineral nutrition also occur (see Stravistava *et al*, 1980).

Fluctuations in the activity level of NR do not always allow a direct conclusion to be drawn about the nature of the underlying control mechanisms involved. This is mainly due to the fact that it is not always possible to determine whether alteration in level of enzyme activity is brought about by changes in the rate of enzyme synthesis and the rate of degradation or by changes in the activity state of pre-existing enzyme through inactivation or reactivation or indeed, a combination of these processes.

It is hoped that by obtaining a better understanding of NR and how it is regulated that ultimately it may be possible to control *in vivo* NR activity in order to exploit nitrogenous reserves at chosen times and thereby influence plant development. In addition, much attention has been paid to a possible use of NR as an index of crop yield in the selection of suitable strains for cultivation (Hageman, 1979).

CLASSIFICATION OF THE ASSIMILATORY NRs

NR from higher plant, fungal and algal sources are described as assimilatory enzymes as they are involved in the assimilation of nitrate into amino acids and proteins. In many bacteria grown under anaerobic conditions NR fulfills a dissimilatory or respiratory role whereby nitrate acts as a terminal electron acceptor in place of oxygen, allowing respiratory generation of ATP. Although the two forms of NR have various characteristics in common

it is outwith the scope of this thesis to discuss them here.

Assimilatory NRs catalyse the reduction of nitrate to nitrite using reduced pyridine nucleotides as cofactors. Three subclasses of NAD(P)H-nitrate oxidoreductase have been distinguished: EC 1.6.6.1 is specific for NADH; EC 1.6.6.2 can use either reduced nucleotide and EC 1.6.6.3 is specific for NADPH. Fungal NRs can only utilise NADPH (Pan and Nason, 1978), *Ankistrodesmus* (Diez *et al*, 1977) and yeast (Guerrero and Gutierrez, 1977) can use either reduced nucleotide with the same effectiveness and while algal and higher plant enzymes normally show a strong preference for NADH, NAD(P)H bispecific NR has been reported in various plant species such as *Lemna minor* (Hageman and Hucklesby, 1971), maize (Campbell, 1978), rice (Shen *et al*, 1976) and soybean (Jolly *et al*, 1976). Wells and Hageman (1974) suggested that the NADPH-NR activity in leaf tissue of certain corn and soybean genotypes was due to conversion of NADPH to NADH by phosphatase(s) in the plant extract during the assay and that NR was actually NADH-dependent. This was shown to be the case in barley (Dailey *et al*, 1982a) but not so in other species where two distinct enzymes, of similar molecular size, have been isolated in soybean (Campbell, 1976) and maize (Campbell, 1978) and partial separation of the two activities has been reported for rice NR (Shen *et al*, 1976).

It is generally believed that NAD(P)H-NRs make use of cytoplasmic NAD(P)H as the reductant but the source of this NAD(P)H is not known with certainty (see Naik *et al*, 1982).

PURIFICATION OF NR

Studies on the molecular properties of NR have been limited due largely to difficulties in obtaining sufficient quantities of homogeneous enzyme. Purification to electrophoretic homogeneity of NAD(P)H-NR was first achieved for the enzyme from *Chlorella vulgaris* (Solomonson *et al*, 1975) using classical techniques. A considerable improvement in yield and speed was, however, achieved with the development of an affinity chromatography procedure for purification of NR namely Blue Dextran Sepharose chromatography (Solomonson, 1975) and subsequently this procedure was successfully applied to purification of NR from many other sources. Affinity chromatography on FAD-Sepharose has also been employed for the purification of fungal NR (Guerrero *et al*, 1977; Pan and Nason, 1978), NADH-Sepharose for the purification of barley NR (Heimer *et al*, 1976) and very recently squash NR has been purified by zinc chelate affinity chromatography (Redinbaugh and Campbell, 1983).

Purification of higher plant NR has proved even more difficult than from other sources due mainly to a greater lability of the enzyme after extraction, even when using stabilising buffer systems (Kuo *et al*, 1980). This has

lead to low recoveries and low specific activities of higher plant NR on purification. While it is possible to achieve specific activities of 125*units/mg protein for *Neurospora* NR (Pan and Nason, 1978) and 86 units/mg protein for *Chlorella* NR (Solomonson, 1975) using Blue Dextran Sepharose chromatography, the highest values reported for higher plant NR so far have been in the range 22-24 units/mg protein for Spinach NR (Notton *et al*, 1977; Guerrero *et al*, 1977) and wheat (Sherrard and Dalling, 1979). Very recently a specific activity of 110 units/mg protein was reported for soybean NR using zinc chelate chromatography but the specific activity of the enzyme after the preceding Blue Dextran Sepharose stage was only 19 units/mg protein (Redinbaugh and Campbell, 1983). However, it has been more usual for values much lower than these to be common, for instance 7 units/mg protein for corn NR (Campbell and Smarrelli, 1978) 8 units/mg protein for barley cv Steptoe (Kuo *et al*, 1980) and 1.9 units/mg protein for the Golden Promise cultivar (Small, 1980), 1.3 units/mg protein for maize scutellum NR (Campbell, 1978) and 1.9 units/mg protein for squash NR (Campbell and Smarrelli, 1978). The reason for the considerably lower specific activities of purified NR for higher plants than *Neurospora* and *Chlorella* is not known.

* 1 unit is defined as 1µmol nitrite formed/min.

PROSTHETIC GROUPS AND ELECTRON FLOW

Flavin

The involvement of a flavin component in nitrate reduction was first suggested by Evans and Hall (1953) who demonstrated that the reduction of nitrate to nitrite by soybean extracts was enhanced by the addition of FAD. The ability of exogenous FAD to stimulate NR activity *in vitro* has subsequently been shown to be species dependent and was found to be an absolute requirement for the expression of NADPH-NR activity of *A. nidulans*. Further, NADH-NR activity of soybean is stimulated by exogenous FAD whereas it is an absolute requirement for the NADPH-NR activity.

Exogenous FAD has been found to stabilise barley NR (Wray and Filner, 1970) and spinach NR (Notton and Hewitt, 1979) during sucrose density gradient centrifugation and also to prevent inactivation of highly purified spinach NR by large dilutions (Notton and Hewitt, 1979).

The enzymatic reduction of exogenous FAD by NADPH in *N. crassa* was shown by Nicholas and Nason (1954a,b,c) to be severely inhibited by the addition of pCMB and reactivated by glutathione, indicating the involvement of sulphydryl group(s). These observations have subsequently been confirmed in higher plants (Sanderson and Cocking, 1964; Schrader *et al*, 1968). It has recently been proposed that sulphydryl groups are directly involved in the electron flow from NADPH to FAD in *N. crassa* (Garrett and Amy, 1978).

Table 1 Absorption Maxima of Reduced and Oxidised Assimilatory NRs

Species	Reduced peaks (nm) α	β	γ (soret)	Oxidised γ	Reference
Hordeum vulgare cv Steptoe	556	528	422	413	Somers *et al* (1982)
Nicotiana tabacum	556	522	418	410	Mendel & Müller (1980)
Spineracia oleracia	560	528	425	415	Notton *et al* (1977)
Neurospora crassa	557	528	423	413	Garrett & Nason (1967)
Aspergillus nidulans	554	524	423	412	MacDonald & Coddington (1974)
Chlorella fusca	557	527	423	412	Solomonson & Vennesland (1972)
Ankistrodesmus braunii	557	527	424	414	De La Rosa *et al* (1980)

Downey (1971) demonstrated that reduced *A. nidulans* NR shows a difference spectral peak at 450 nm which was indicative of FAD. Reoxidation of FAD by the addition of nitrate was inhibited by potassium cyanide but not by pCMB suggesting that the sulphydryl groups are located prior to the FAD binding site.

Notton *et al* (1977) have confirmed that FAD is the flavin component of spinach NR using both spectral observations and a specific biochemical test based on the reactivation of apo-D-amino acid oxidase (De Luca *et al*, 1956).

Haem

Cytochrome involvement in assimilatory nitrate reduction was first demonstrated by Garrett and Nason (1967) who showed specific reduction by NADPH of a *b* type cytochrome (designated cyt b_{557}) which was reoxidised by nitrate thus indicating that the cytochrome was an integral part of the electron transport sequence of the enzyme. The same authors showed that although the reduction of cyt b_{557} was dependent on the presence of exogenous FAD, reoxidation was not. This suggested to them that the FAD component of the enzyme must be located between the NADPH binding site and the cyt b_{557}, thus cyt b_{557} accepts electrons from FAD.

Functional cytochrome b_{557} has now been identified as a component of NR from many sources (Table 1). Minor differences in the values for the absorption maxima being in some part attributable to measurement at different temperatures.

Low temperature epr signals of purified *N. crassa* NR revealed $g = 2.98$ and $g = 2.27$ resonances which are consistent with a low spin haem iron complexed to two imidazole residues which is typical of *b*-type cytochromes (Garrett and Amy, 1979).

Molybdenum

The presence of molybdenum (Mo) as a component of higher plant NR has been demonstrated from deficiency experiments (Steinberg, 1937; Hewitt, 1951) and tungstate (WO_4) antagonism studies. Heimer *et al* (1969) showed that growth in the presence of W (an analogue of Mo) prevented the formation of an active NR and Wray and Filner (1970) went on to show that in the presence of W an intact, but inactive, NR molecule was formed. Notton and Hewitt (1971c) demonstrated ^{185}W labelling of spinach NR when induced by nitrate in the presence of W, thus providing direct evidence for the formation of a tungsto-protein analogue of NR. The presence of Mo in spinach NR was also demonstrated directly by the same authors with the use of ^{99}Mo in molybdenum deficient plants (Notton and Hewitt, 1971a).

Experimental evidence, obtained mainly from work with fungal systems, has shown that Mo is associated with a dialysable component with MW 1 000 - 1 500 (Lee *et al*, 1974). This Mo component (MoCo) has been shown to be non-covalently attached to NR (Nason *et al*, 1971; Johnson *et al*, 1977). The non-covalent binding of MoCo

is illustrated in higher plants by its ease of separation from the enzyme during contact with AMP- and Blue Sepharose (Hewitt *et al*, 1979) and its ability to reconstitute the holoenzyme when mixed *in vitro* with the apoenzyme (Rucklidge *et al*, 1976).

Isolated MoCo is highly sensitive to oxygen and MoCo isolated in the inactive form has been shown to contain a novel pteridine as a structural component (Johnson *et al*, 1980).

MoCo would appear to exist in a bound form as acid treatment (Mendel *et al*, 1981) or heat treatment (Mendel *et al*, 1982) is required for maximal release of MoCo from tobacco cell NR. The MoCo isolated from *E. coli* NR is insensitive to trypsin but it is associated with a carrier molecule which is easily removed by dialysis. The carrier molecule provides protection of the cofactor from inactivation by heat or oxygen (Amy and Rajagopalan, 1979).

With the exception of nitrogenase (Shah and Brill, 1977; Pienkos *et al*, 1977) it would appear that MoCo is present in a similar, if not identical, form in molybdenum enzymes, catalysing diverse reactions from bacterial, plant and animal sources (Johnson, 1980).

The molybdenum domain of NR is thought to be the site where nitrate binds to the enzyme and is reduced to nitrite. Evidence for the direct involvement of molybdenum at the active site of the enzyme comes from

several observations. Nicholas and Nason (1954a) demonstrated that dithionite reduced sodium molybdate could serve anaerobically as an electron donor for *N. crassa* NR in the absence of added FAD thus suggesting that the molybdenum site is located closer to the active site of the enzyme than is the flavin site. In addition, the metal binding agents such as cyanide and azide inhibit nitrate reduction regardless of electron donor (see following section).

The sequence of electron flow from NAD(P)H to nitrate in assimilatory NRs is generally accepted to be:-

$$NAD(P)H \xrightarrow{-SH} FAD \longrightarrow \text{cyt } b_{557} \longrightarrow Mo \longrightarrow NO_3^-$$

REACTIONS CATALYSED BY NR *IN VITRO*

In addition to catalysing the physiological reaction, NR can also reduce nitrate *in vitro* using $FMNH_2$, $FADH_2$ or reduced methyl or benzyl viologen dyes as electron donors (Cresswell *et al*, 1965; Schrader *et al*, 1968; Wray and Filner, 1970). NR also exhibits a NADH-dehydrogenase function whereby it can donate electrons to reduce cytochrome *c* (Wray and Filner, 1970), DCPIP (Nicholas and Nason, 1954b), ferricyanide, tetrazolium salts (Pateman *et al*, 1967) and benzoquinone and menadione (Smarrelli and Campbell, 1979).

SITES OF INTERACTION OF ELECTRON DONORS AND ACCEPTORS WITH NR

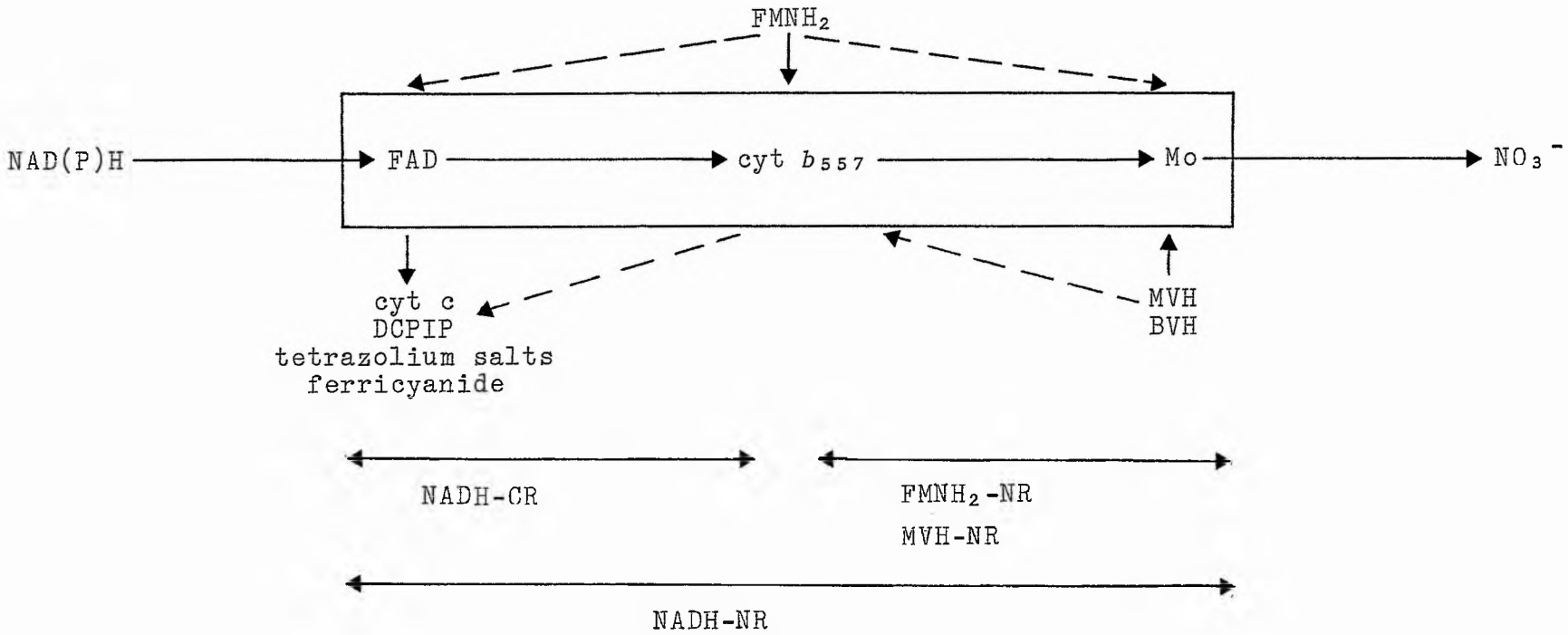
The probable sites of interaction of electron donors and acceptors with NR are outlined in Fig.2. The evidence for the scheme obtained mainly from heat inactivation and inhibitor studies is detailed below.

The different enzyme activities expressed by the NR complex exhibit differential stabilities to heat inactivation. $FMNH_2$-NR was shown to be more heat stable than NADH-NR and NADH-CR activities suggesting that the component involved in binding NADH was less stable than that binding $FMNH_2$ (Schrader *et al*, 1968; Wray and Filner, 1970). In contrast Sherrard and Dalling (1979) considered the site of acceptance of electrons from NADH to be the most stable portion of highly purified wheat NR and showed that it could not be due to a highly stable contaminant with NADH-ferricyanide reductase activity. The extent of differential instability is dependent on the presence of exogenous FAD in the medium since FAD has been shown to protect NR against heat inactivation in *Chlorella* (Zumft *et al*, 1970), spinach (Relimpio *et al*, 1971) and maize (Roustan *et al*, 1974).

Treatment with the thiol-blocking reagent pCMB markedly affects the NADH-NR and NADH-CR activities of the complex while $FMNH_2$-NR and MVH-NR activities are less sensitive (Wray and Filner, 1970). On the other hand, treatment with azide, cyanide or organic chelating agents

Fig.2

Possible sites of interaction of substrates and electron donors with NR



result in inhibition of all NR activities except NADH-CR activity (Hageman and Hucklesby, 1971; Smarrelli and Campbell, 1979).

The differential effects of the various inhibitors, together with susceptibility to heat treatment, suggest that NR can functionally be subdivided into two distinct parts: a proximal NADH-oxidising region responsible for NADH-CR activity and a nitrate-reducing region responsible for $FMNH_2$-NR and MVH-NR activities.

It is not clear whether cyt b_{557} is obligatorily involved in the expression of CR activity. When the reduced spinach enzyme was titrated with DCPIP to a faint blue end point the Soret band was shown to be in the oxidised form (415 nm) but changes at 560 nm were masked by DCPIP indicating some haem involvement with the dehydrogenase function either directly, or possibly by some back electron flow mechanism (Notton *et al*, 1977). Determination of the mid-point potential of the haem component of spinach NR did not help to resolve this issue (Fido *et al*, 1979). However, in *Neurospora* cyt b_{557} is reduced by NADPH only when FAD is present (Garrett and Nason, 1969). It is clear though that molybdenum is not required for NADH-CR activity and FAD is not required for MVH-NR activity.

The fact that MVH-NR activity is more heat stable than $FMNH_2$-NR (Garrett and Nason, 1969; Wray *et al*, 1979) coupled with the fact that MVH-NR and $FMNH_2$-NR

activities behave differently in the presence of SH-group inhibitors (Mendel and Muller, 1980), suggest that they do not donate electrons at the same site. As $FMNH_2$-NR activity is less heat stable than MVH-NR activity this suggests that reduced viologen dyes donate electrons to a site closer to the nitrate reducing site than that to which $FMNH_2$ donates. It is believed that as reduced viologen dyes have a high electronegative potential, they donate electrons directly to the Mo site (Hewitt *et al*, 1976). Small (1980) observed a biphasic response of $FMNH_2$-NR activity to heat treatment and suggested that $FMNH_2$ may be capable of donating electrons to two sites with different heat stabilities.

As a result of studying the stability of the activities of highly purified wheat leaf NR Sherrard and Dalling (1979) have proposed a novel model for NADH-NR which incorporates two separate pathways of electron flow to nitrate. Each pathway is envisaged to be distinct with respect to pH optimum, electron donor affinity and temperature sensitivity. NADH would have equal access to either pathway but one pathway could receive electrons from MVH and the other from $FMNH_2$.

KINETIC MECHANISMS OF NR

There is no general agreement to the kinetic mechanism of NR from various sources, which is surprising since all assimilatory NRs contain identical prosthetic groups and catalyse identical partial reactions.

The enzymes from corn and squash (Campbell and Smarrelli, 1978), spinach (De la Rosa and Palacian, 1981) and *Ankistrodesmus* (Herrero *et al*, 1980) yield parallel NAD(P)H/nitrate initial velocity reciprocal plots which, together with the expected product inhibition patterns, suggest a non-classical, two-site, hybrid ping pong rapid equilibrium random mechanism. An ordered mechanism has, however, been suggested for the spinach enzyme by Eaglesham and Hewitt (1975) in which NADH is oxidised and released as NAD^+ before the attachment of nitrate which is then reduced to nitrite. The enzymes from *Chlorella vulgaris* (Howard and Solomonson, 1981) and *Aspergillus nidulans* (MacDonald and Coddington, 1974) yield intersecting NAD(P)H/nitrate plots, which together with the expected product inhibition data suggest a classical rapid equilibrium random mechanism.

It is possible that the enzymes from different sources do have intrinsically different mechanisms. However, it is also possible that native NRs from all sources have the same kinetic mechanism but structural modification of the enzyme during purification or storage occurs which causes the ratios of certain rate constants to change (Renosto *et al*, 1982).

MOLECULAR WEIGHTS AND SUBUNIT STRUCTURE OF HIGHER PLANT NRs

Sucrose density gradient analysis of crude extracts of barley revealed 3 types of NADH-CR activity (Wray and Filner, 1970). The heaviest one of these, sedimenting at the bottom of the gradient, was observed in extracts from both nitrate-grown and nitrate-less plants and is likely to be of microsomal origin and therefore equivalent to the constitutive NADPH-CR species found in fungal extracts (Sorger, 1966). The 8S CR species which co-sedimented with NADH-, $FMNH_2$- and MVH-NR activities represents the intact NR complex and was found only in extracts from nitrate-grown plants. The third CR species which sedimented at 3.7S was found in extracts from nitrate-grown and nitrate-less plants but the activity associated with this species was approximately two-fold higher in extracts from nitrate-grown plants. It was postulated by Wray and Filner (1970) that the 3.7S CR species was either a dissociation product of NR or a precursor protein for the enzyme.

The use of much larger sucrose gradients than those used by Wray and Filner (1970) and comparison with reference proteins catalase, alcohol dehydrogenase and myoglobin has enabled Small (1980) to make more accurate measurements of the sedimentation coefficients of the CR species observed in extracts of barley leaves. The Stokes radii of the NR and CR species were also determined by Sephadex gel filtration and the molecular weight and frictional

ratio (Siegel and Monty, 1966) and axial ratio (Oncley, 1941) of these species calculated. Nitrate-less plant extract possessed one small CR species of MW 27 800 (2.71S). While nitrate grown plant extracts also possessed this species, its amount was not affected by the presence of nitrate and it was therefore assumed to be unrelated to NR. The NR complex which was present only in nitrate-plant extracts had a sedimentation coefficient of 7.7S, a Stokes radius of 6.4 nm giving a calculated MW of 203 000 and a frictional ratio(f/fo) of 1.65. The molecule is highly asymmetrical with an axial ratio (r_1/r_2) of 11:1. Two further small CR species which were present only in nitrate-plant extracts had sedimentation coefficients of 3.8S and 3.1S, stokes radii of 3.9 nm and 3.1 nm and MWs of 61 000 and 40 000 respectively. These species were thought likely to be derived from NR since sucrose density gradient analysis of a sample from the leading edge of partially purified NR peak eluted from Biogel A 1.5 m (which would be expected to contain species of 200-250 000 MW) revealed the presence of relatively large amount of 3.8S and 3.1S species (but not 2.7S species) which were presumably derived from unstable NR molecules. In older tissues CR species sedimenting at 5.6S and 6.8S were observed, the 6.8S species also possessing NR activity. These species were at first thought to represent subunits or associations of subunits of the enzyme (Wray *et al*, 1979) but it was later postulated that the species might represent fragments released from the enzyme as a result of the action of endoproteinases (Small and Wray, 1980a).

Notton *et al* (1976) revealed two peaks of NADH-CR activity sedimenting at 8.1S and 3.7S on subjection of extracts of leaves from nitrate grown spinach plants to sucrose density gradient analysis, thus confirming the results of Wray and Filner (1970) with barley leaf extracts. The two species were demonstrated to be related by nitrate starvation and replenishment studies (Notton *et al*, 1976). Additional peaks of CR activity occurring at 5.5S and 6.9S, neither of which possessed NR activity, were observed in extracts of plants grown with or without tungsten but with ammonium as an additional nitrogen source to nitrate (Notton *et al*, 1976).

Wallace and Johnson (1978) reported that the so called 4S CR species commonly found in higher plant extracts actually consists of at least 3 species. In contrast to Small, however, these authors concluded that only one of the species was related to NR since only one was sensitive to attack by a NR-specific proteinase isolated from maize root (Wallace, 1975b) and that this species alone might represent the subunit of NR.

Another CR species observed in extracts of cotton and other plant species, including barley, was found only to be active in the presence of ferrocyanide (Wallace and Johnson, 1978). The presence of this ferrocyanide-activated CR species in barley leaf extracts was confirmed by Small and Wray (1980b). The enzyme had a calculated

molecular weight of 45 000, a frictional ratio of 1.19 and an axial ratio of 3.1. The fact that this species was more globular than NR-related CR species and that it was present in extracts from both nitrate and nitrate-less plants suggested to the authors that ferrocyanide-activated CR is unrelated to NR.

Multiple bands were obtained on SDS gel electrophoresis of purified barley NR by Small (1980), one of which corresponding to MW 105 000 was thought to be the intact subunit of NR. It was thus concluded that barley NR was a homodimer. Work subsequently carried out in the same laboratory (J. McA. Campbell and Wray, 1983) using the same cultivar of barley confirmed the native MW of approximately 205 000 by electrophoresis of purified NR on different percentage gels and the subunit MW of approximately 103 000 by SDS gel electrophoresis. In addition, the ratio of bands corresponding to values less than 105 000 MW was found to be dependent on the purification conditions, details of which will be given in the General Discussion.

Kuo *et al* (1980) have recently reported the purification of NR from the barley cultivar Steptoe using a specially developed stabilising buffer (see Results Chapters 2 and 3). From a sedimentation coefficient of 7.9S and a Stokes radius of 6.8 nm a MW for the native NR of the barley cultivar Steptoe was calculated to be 221 000. SDS gel electrophoresis indicated a subunit MW of approximately 100 000 and NR was concluded to be a homodimer.

SDS gel electrophoresis of purified spinach NR revealed multiple bands, some of which corresponded approximately to nominal molecular weights designated to the CR species observed on sucrose density gradient (Notton and Hewitt, 1979). From these data it was therefore proposed that spinach NR was a tetramer with a monomeric MW of 37 000 (Notton *et al*, 1979). However, these were not valid conclusions to draw as the same authors (Notton *et al*, 1976) had shown spinach NR to be highly assymetrical with an axial ratio of 10:1 indicating that molecular weight determination based only on sucrose density gradient to be erroneous and a molecular weight of 197 000 had been calculated from the sedimentation coefficient of 8.1S and the Stokes radius of 6 nm (from gel filtration). The Long Ashton group, however, no longer favour a tetrameric structure for spinach NR since subsequent, as yet unpublished, work suggests the enzyme to be a homodimer (B.A. Notton, personal communication).

Recently Campbell (1982) showed squash NR to have a native MW of 230 000 by analysing purified enzyme on differing polyacrylamide concentrations. SDS gel electrophoresis revealed two polypeptides with MW 44 000 and 75 000 or one polypeptide with MW 110 000 depending on the purification procedure. They concluded that as 40 000 MW FAD dehydrogenases are commonly observed in higher plant extracts (see earlier) the 44 000 MW polypeptide is probably equivalent to the dehydrogenase portion of NR while the cytochrome-Mo portion is made up

of a 75 000 MW polypeptide plus some form of MoCo. The 110 000 MW species was considered to be an adduct of the two smaller polypeptides and likely to be the true subunit.

It can be concluded from data described here for higher plant NR that the enzyme has an approximate molecular weight of 200 000 and is composed of two identical subunits of approximately 100 000. Flavin stochiometry has not been determined due to the ease of FAD dissociation during purification but the haem to molybdenum ratio for the spinach enzyme is approximately 2:1 (Notton and Hewitt, 1979). Since the subunits are likely to be identical it has been assumed that there are 2 mol FAD/mol enzyme. NR is therefore envisaged to consist of two haemoflavoprotein subunits with a low molecular weight MoCo of the type identified in other molybdoenzymes (Johnson *et al*, 1980; Rajagopalan *et al*, 1980).

However, the ubiquitous nature of the small, apparently NR-related NADH-CR species observed in plant extracts and the frequently observed approximately 40 000 and 60 000 MW protein species obtained on SDS gel electrophoresis of purified enzyme from a variety of sources must be accounted for in the structure of NR.

To determine whether a similar phenomenon also exists in other systems, a short review of the molecular weights and subunit structures of various algal, fungal and yeast NRs will be undertaken.

MOLECULAR WEIGHTS AND SUBUNIT STRUCTURE OF NRs FROM NON-PLANT SOURCES

Chlorella

The most extensive structural studies have been carried out on NR from *C. vulgaris* as the organism lends itself well to such studies because of the high content and relative stability of the enzyme in crude cell extracts (Solomonson *et al*, 1975; Giri and Ramadoss, 1979).

Molecular weights calculated for the *Chlorella* enzyme have been consistently higher than those from higher plants: 356 000 (Solomonson *et al*, 1975), 280 000 (Giri and Ramadoss, 1979) and 360 000 (Howard and Solomonson, 1982). SDS gel electrophoresis of purified *Chlorella* NR showed one protein staining band corresponding to a molecular weight of 100 000 (Solomonson *et al*, 1975) or 90 000 (Giri and Ramadoss, 1979). Quantitative analysis of prosthetic groups revealed FAD : haem : molybdenum ratios of 2.35:2.35:1.92 (Solomonson, 1975) and 2.58:2.50:2.05 (Giri and Ramadoss, 1979). This data was interpreted to indicate that the *Chlorella* enzyme is a trimer of identical subunits, each possessing all three prosthetic groups.

Howard and Solomonson (1982) have recently provided evidence, from cross-linking experiments with dimethyl suberimidate, that the *Chlorella* enzyme is a tetramer. This is, however, difficult to reconcile with previously

published results for stochiometry of the prosthetic groups, especially if the enzyme is a homotetramer which the same authors indicated from tryptic mapping of the subunits. It was also shown that *Chlorella* NR would appear to dissociate at low protein concentration into a dimeric form which has the same specific activity as the tetrameric species.

Ankistrodesmus braunii

The NR from *A. braunii* has an estimated molecular weight of 467 400 (De La Rosa *et al*, 1981) and is thought to be composed of 8 identical subunits of molecular weight 58 750 (De La Rosa *et al*, 1980; 1981). This homopolymeric model for the enzyme is, however, not consistent with the results from analysis of prosthetic group content namely 4 mol FAD, 2 mol molybdenum and 4 mol haem/mol enzyme.

Neurospora crassa

A molecular weight of 228 000 has been determined for *N. crassa* NR (Garrett and Nason, 1967; 1969) which more closely resembles that from higher plants than algal sources. SDS gel electrophoresis of the purified enzyme revealed two protein bands corresponding to molecular weights of 115 000 and 130 000 suggesting the existence of two kinds of subunit. However, proteolytic mapping of the two protein bands showed identical patterns and

only one N terminal amino acid was detected (Pan and Nason, 1978). Since variable ratios of the two bands were observed with different preparations of the purified enzyme it was suggested that the two subunits were either converted from one to the other or derived from a single subunit.

The enzyme was shown by Pan and Nason (1978) to contain 1.54 mol haem and 0.89 mol molybdenum/mol enzyme which was taken to be equivalent to a ratio of haem : molybdenum of 2:1. FAD content was not determined due to its ease of dissociation but was assumed to be 2 FAD/mol enzyme. From this data the authors proposed that *N. crassa* is a dimer of 115 000 dalton subunits, each possessing FAD and cyt b_{557} linked together with a MoCo.

Recently, Tachiki and Nason (1983), obtained a molecular weight of 272 000 for *N. crassa* NR by employing a shortened purification procedure which minimised proteolysis thus suggesting that the 130 000 dalton band observed by Pan and Nason (1978) was more likely to represent the true subunit.

Aspergillus nidulans

Molecular weights of 197 000 (Downey, 1971) and 190 000 (MacDonald and Coddington, 1974) have been calculated for the native *A. nidulans* NR. Downey and Focht (1974) showed the presence of only one band on SDS gel electrophoreses which corresponded to a molecular

weight of 49 000 thus suggesting a tetrameric structure for the enzyme. In contrast, Downey and Steiner (1979) reported that the *A. nidulans* enzyme dissociated into two different subunits of 75 000 and 50 000 daltons from either a 240 000 or a 118 000 dalton native NR complex. Such data could be interpreted as indicating that the 118 000 molecular weight species is the subunit of NR and the 75 000 and 50 000 dalton species were degradation products of it. However, both 118 000 and 240 000 dalton species possessed BVH-NR-staining activity on gels which is not consistent with the 1 mol molybdenum/mol enzyme calculated by Downey (1973a). One mol FAD/mol enzyme was also calculated by Downey (1973a) but as the FAD is known to be only loosely associated with the fungal enzyme this value is likely to be an underestimate. Due to the controversy over whether (Downey and Steiner, 1979) or not (Downey, 1971) the *A. nidulans* NR contains haem, cyt b_{557} stochiometry remains unknown.

Evidence for modification of the native NR *in vitro* comes from the observation that although small NADPH-CR species of approximately 4.5S (of the type commonly observed in mutant extracts) were not normally present in wild type extracts they were shown to be produced on storage of the extract suggesting that these species were generated *in vitro* as a result of proteolytic degradation.

Heat treatment has also been shown to affect structural modifications of the enzyme. Minagawa and

Yoshimoto (1982) have recently reported that *A. nidulans* NR comprises of two subunits of molecular weight 59 000 and 38 000 but that dissociation of the 38 000 subunits from the native enzyme occurs during heat treatment.

Rhodotorula glutinis

The structure of NR from the false yeast *Rhodotorula glutinis* would appear to be similar to that of higher plants and fungi, having a molecular weight of 230 000 with subunits of 115 000 or 118 000 daltons (Guerrero and Gutierrez, 1977). Non-denaturing gel electrophoresis of the enzyme revealed two protein staining bands which both exhibited NADPH-dehydrogenase and MVH-NR activities. The major band contained over 90% of the total protein but the relative amount of protein associated with the fainter band was shown to increase upon storage and it was suggested that the minor component was a degradation product of the native enzyme.

Haem stochiometry of the yeast NR was determined relative to *C. vulgaris* NR and shown to be 70% of the amount present in the algal enzyme. This was interpreted by Guerrero and Gutierrez (1977) to indicate 1 mol haem/mol enzyme thereby concluding that *R. glutinis* NR subunits were the same size but were not identical. However, on the basis of a value of 2.34 mol haem/mol *C. vulgaris* NR, Solomonson *et al* (1975) suggested that the algal NR contained 3 mol haem/mol enzyme, i.e. 1 mol haem/mol

subunit, therefore it is likely that the *R. glutinis* enzyme also contains 1 mol haem/mol subunit.

From the data available, from all systems described here, there would appear to be abundant evidence to support the conclusion that NR is susceptible to modification on extraction, thereby leading to confusion with regard to estimation of molecular weights and subunit composition.

BIOCHEMICAL GENETICS

Fungi

Mutants deficient in NR activity have proved important tools for studying biochemical aspects of nitrate assimilation. An appreciable body of information exists on the biochemical genetics of fungi due to the fact that it is easy to isolate mutants from, and carry out genetic analyses on these organisms.

In *A. nidulans* mutations in at least 17 loci can lead to an impaired ability to use nitrate as the sole source of nitrogen. MacDonald and Cove (1974) showed that a temperature sensitive mutation in *nia* D resulted in a NR which was more labile than the wild type. As altered thermal stability of a protein is often thought to be a good indication that alteration of the primary structure of the protein has taken place this provided evidence for the thesis of Pateman *et al* (1964) that the

nia D gene codes directly for a structural component of NR.

Mutations in at least 5 genes, designated *cnx*, were found to result in loss of ability to grow on hypoxanthine (Pateman *et al*, 1964) and have subsequently been identified as MoCo mutants.

Several *Aspergillus* mutants lacking NADH-NR activity have been observed to possess a species of NADH-CR activity which sedimented at 4.5S on sucrose gradients. This species was thought likely to represent either a subunit or breakdown product of NR (MacDonald *et al*, 1974). The only mutant which never possesses this small NADH-CR species is *cnx* E (Downey, 1973b; MacDonald *et al*, 1974) which was presumed to possess a structurally intact MoCo since it was repairable by growth in the presence of high concentrations of molybdate (Arst *et al*, 1970). MacDonald *et al* (1974) proposed that the 4.5S CR species was the product of the *nia* D gene. The same authors estimated the molecular weight of the 4.5S species to be 100 000 and therefore proposed that *A. nidulans* NR was a dimer of the 100 000 MW *nia* D gene product and that dimerisation was brought about by the presence of intact MoCo. However, the MW of this 4.5S CR species is in dispute. Lewis (1975) estimated its MW to be approximately 50 000 and suggested that the 7.6S enzyme was an aggregate of 4 4.5S products. In addition Lewis (1975) described some heterogeneity of the so-called 4.5S CR species. Although these species were not normally detected in

extracts from wild type mycelia, they were shown to be produced on storage of the extract suggesting that these species are generated, *in vitro*, as a result of proteolytic degradation of some larger gene product (presumably *nia* D). Garrett and Cove (1976) showed that the level of NR activity which could be reconstituted by co-homogenisation of *nia* D and *cnx* mutant mycelia could be doubled in the presence of PMSF (an inhibitor of serine proteinases) thus suggesting that *A. nidulans* NR is susceptible to attack by endogenous proteinases.

Sorger (1966) described two classes of *N. crassa* mutant, *nit* 1 and *nit* 3 that coded for structural components of NR. The *nit* 1 produces an enzyme which possesses nitrate-inducible NADPH-CR activity, but no other related NR activities while the *nit* 3 mutant has $FMNH_2$-NR and MVH-NR activities but no NADH-NR or NADH-CR activities. The *nit* 1 gene is thought to code for MoCo (analagous to *cnx* genes of *A. nidulans*) and the *nit* 3 gene to code for the rest of the NR molecule (analagous to the *nia* D gene of *A. nidulans*).

CR species derived from *nit* 1 mutants have been shown to have sedimentation coefficients in the range 4.5S-5.2S (Nason *et al*, 1970; Ketchum and Downey, 1975). The *nit* 1 enzyme has proved difficult to purify, mainly because of its instability, but estimates of 50 000 (Nason *et al*, 1970), 84 000 (Coddington, 1976), 115 000 (Pan and Nason, 1978) and 130 000 (Ketchum and Downey, 1975) have been reported. However, recently Horner (1983), employing

a shortened procedure for purification of *nit* 1 enzyme which minimised proteolysis, obtained one major protein band on SDS gel electrophoresis corresponding to a MW of 145 000. Using a shortened procedure for partial purification of wild type *N. crassa* NR, Tachiki and Nason (1983) obtained a MW of 272 000 for the native enzyme which is larger than previously reported by Garrett and Nason (1969). It was concluded that the *nit* 1 enzyme is the apoenzyme of NR and that the wild type enzyme is a homodimer of 145 000 subunits.

Antoine (1974) purified NR from the *nit* 3 mutant of *N. crassa*. The enzyme was found to contain cyt b_{557}, have a sedimentation coefficient of 6.8S and MW of 160 000. Using the shortened purification procedure described above for the wild type enzyme, Tachiki and Nason (1983) found that the MW of the *nit* 3 enzyme was 204 000. In addition the *nit* 3 subunit had a MW of 98 000, suggesting that the *nit* 3 enzyme also consists of two identical subunits. Using either the long purification procedure described by Antoine (1974) or the shortened version described by Tachiki and Nason (1983) it is clear that the *nit* 3 enzyme is smaller than that of the wild type enzyme. It is possible that the *nit* 3 mutant is a dimer of a deletion, or nonsense mutant which can still interact with cyt b_{557} and MoCo to give a partially functioning enzyme. Alternatively, it may represent a dimer of a proteolytic fragment generated by proteinase action on an aberrant *nit* 3 gene product possibly resulting in the loss of the FAD- containing region but still allowing

association to take place.

Algae

Despite the large amount of biochemical data accumulated for *Chlorella* NR, very little genetic analysis has been undertaken with this species. However, Sosa *et al* (1978) obtained 4 classes of mutants of *Chlamydomonas reinhardii* by exposure of the cells to N-methyl-N'-nitro-N-nitrosoguanidine and subsequent selection for chlorate resistance. One class of mutant expressed no NR-associated activities and was therefore thought likely to have undergone mutation in a regulatory gene for NR while another class of mutant had normal *in vitro* levels of NR activities but could not grow on nitrate indicating that nitrate uptake may have been affected. Two sets of mutants contained intermediate levels of NR activities compared with the wild type. The first class showed appreciable NADH-CR activity with a sedimentation coefficient of 3.5S but lacked all other NR activities and would therefore appear to be analagous to some of the *cnx* mutants of *A. nidulans* and to the *nit* 1 mutant of *N. crassa*. The second class of mutant had no CR activity but high levels of $FMNH_2$-NR and MVH-NR activity which sedimented at 8.3S on sucrose gradient (compared with 10S for wild type enzyme) and is likely to be analagous to the *nit* 3 mutant of *N. crassa*.

In vitro complementation of NAD(P)-NR has been achieved by mixing cell-free extracts of *Chlamydomonas*

reinhardii mutant 104 (possessing 3.5S NAD(P)H-CR activity) and mutant 305 (possessing 8.3S $FMNH_2$-NR activity) (Fernández and Cardenas, 1981). From the biochemical characterisation of the mutant enzymes and the mode of *in vitro* complementation, it has been suggested that the NR of *C. reinhardii* is a heteromultimer consisting of at least two types of subunit, separately responsible for the proximal and terminal activities of NR (Fernández and Cardenas, 1981, 1982 , 1983c).

Higher Plants

Genetic studies are made difficult in higher plants due to the inherent problems in selecting and propagating specific metabolic mutants. However, mutants have relatively recently been identified and characterised in *Arabidopsis thaliana* (Oostindier-Braaksma and Feenstra, 1973), *Datura innoxia* (King and Khanna, 1980), *Pisum sativum* (Feenstra and Jacobsen, 1980; Kleinhofs *et al*, 1978; Warner *et al*, 1982), *Nicotiana tabacum* (Muller and Grafe, 1978; Mendel and Muller, 1979; 1980; Buchanan and Wray, 1982) and *Hordeum vulgare* (Warner *et al*, 1977; Kleinhofs *et al*, 1980; Kuo *et al*, 1981; Somers *et al*, 1983a; Bright *et al*, 1983). Only in the latter two species has an extensive investigation of the biochemical properties of the mutants been carried out.

Mutagenesis of *N. tabacum* cell lines and subsequent selection for chlorate resistance has produced NR-negative mutants (Muller and Grafe, 1978) which have been subjected to enzymatic characterisation (Mendel and Muller,

1979). All mutants which were defective in the apoprotein of NR were shown to be allelic, representing mutations in the structural gene for NR (designated *nia* D). The remaining mutants were found to be MoCo mutants (analagous to the *cnx* mutants of *A. nidulans* and *nit* 1 mutant of *N. crassa*).

Mendel and Muller (1980) compared the properties of affinity purified NR from wild type and MoCo defective *cnx*-68/2 cell line of *N. tabacum*. They found that kinetic parameters, inhibitor specificities, haem content, heat stability and sedimentation coefficients were almost identical, showing that the apoprotein of the *cnx* mutant NR is unaffected by the mutation and the defect resides in the MoCo.

All of the *cnx* mutants isolated by the Gaterslaben group have been shown to contain a MoCo which is active in complementing *N. crassa nit* 1 mutant in a reaction which is completely dependent on the addition of 10-30mM molybdate. This suggests that the *cnx* mutant NR possesses a MoCo defective in catalytic properties but is still able to mediate the assembly of subunits and is therefore equivalent to the *cnx* E mutants described in *Aspergillus* (Pateman *et al*, 1964; Arst *et al*, 1970).

Mendel and Muller (1980) also showed that upon affinity chromatography a portion of the NR (7.6S) molecules, from both the wild type and *cnx* mutant line, dissociated into CR-active species (4.1S), assumed to be

the NR subunit. Sucrose density gradient sedimentation profiles of NADH-CR activity of both wild type and mutant NR revealed that the ratio of the NADH-CR peaks at 4.1S and 7.6S varied, in a reciprocal manner, from purification to purification, suggesting that the enzyme is susceptible to modification *in vitro*.

Buchanan and Wray (1982) have isolated a second phenotypic class of MoCo mutant in *N. tabacum* (*cnx* B) which could not be repaired *in vitro* by molybdate but which was active in complementing *N. crassa nit* 1 mutants *in vitro* (Mendel *et al*, 1984). A third phenotypic class, *cnx* C, isolated from *N. plumbagimfolia* could neither be repaired by molybdate or complement *nit* 1 *in vitro* (Mendel *et al*, 1982).

Ten NR-deficient mutants have been isolated from azide mutagenised *Hordeum vulgare* cv Steptoe (Warner *et al*, 1977). Genetic analysis of the mutants indicated that nine of the mutants were allelic (*nar* 1a-*nar* 1i) and one mutant represented a separate NR gene (*nar* 2a) (Kleinhofs *et al*, 1980). A further three NR deficient barley mutants (*Xno* 18, 19, 29) have been isolated by Tokarev and Shumney (1977) using EMS mutagenisis and selection for chlorate resistance. As the mutants *Xno* 18 and *Xno* 19 are allelic to one another but not to *nar* 1 or *nar* 2, Kleinhofs *et al* (1983) proposed this locus be designated *nar* 3. *Xno* 29 is however allelic to *nar* 1 and is therefore the 10th allele at this locus and is designated *nar* 1j. In the presence of nitrate all of the NR-deficient

mutants described (except *Xno* 29 and *nar* 1j) have elevated levels of nitrite reductase but possess different levels of NR-associated enzyme activities. The *nar* 1d (Az 28) mutant contains very high levels of NADH-CR activity while *nar* 1h (Az 32) has above normal $FMNH_2$-NR activity (Kleinhofs *et al*, 1980) and is temperature sensitive (Somers *et al*, 1983a). This suggests that the *nar* 1d and *nar* 1h mutants have NR present in altered forms. As all of the *nar* 1 mutants are allelic it follows that the *nar* 1 locus represents the structural gene for the NR protein. The mutants *nar* 2 and *nar* 3 lack xanthine dehydrogenase activity as well as NR activity and are thus presumed to be MoCo mutants.

The defective NR protein possessed by the NR deficient mutants has also been assessed by immunological methods and antigenicity was found to correlate well with partial NR activities (Kuo *et al*, 1981).

The *nar* mutants possessing significant amounts of NADH-CR activity were found to fall into two classes with respect to proportion of 8S and 4S CR species in extracts. In extracts of mutants *nar* 1b, 1g and 1ℓ, most of the CR was in the 4S form while in *nar* 1d and 2a the CR was mostly in the 8S form. However, in the latter group the proportion of 4S to 8S CR was found to be dependent on *in vitro* conditions thus suggesting that mutant NR protein is susceptible to modification *in vitro*. Subsequent to the completion of the work to be described in this thesis, Kleinhofs' group have intensively investigated the nature

of the 4S CR species observed in mutant and wild type extracts of barley or Steptoe. The results of these studies will be analysed in the General Discussion section of this thesis, in relation to the findings to be described here for barley cv Golden Promise.

Since it appears that the study of the wild type enzyme from various sources is complicated by modification of the enzyme *in vitro* it is not surprising to also find evidence for modification of mutant NR *in vitro*. Before the biochemical characterisation of mutants can be definitively carried out it is therefore essential to stabilise NR protein *in vitro*.

FACTORS AFFECTING NR ACTIVITY *IN VITRO*

Studies on the mechanism and regulation of nitrate reduction have also been hampered by the questionable significance of enzyme assay procedures applied to crude extracts. This problem has not entirely been resolved by application of *in vivo* assay procedures which involve measurements of nitrite excreted by intact tissue after various prior treatments (Jaworski, 1971; Jones *et al*, 1977) as it is generally observed that greater enzyme activities can be obtained using an *in vitro* assay than an *in vivo* assay. The ratio between the activities obtained by the two techniques have, however, been shown to be dependent on the source and age of the tissue.

Wallace (1975a) showed that *in vitro* assay of NR from the tip region of the primary root of maize seedlings indicated a five-fold greater activity than that obtained with an *in vivo* assay while in a more mature region of the root the ratio of *in vitro* to *in vivo* activity was much lower and in older seedlings was less than unity. These observations were taken to indicate that NR became labile on extraction, especially in older tissue and that the nitrate-reducing capacity of such tissue is likely to be under-estimated using an *in vitro* assay.

Schrader *et al* (1974a) also investigated this instability phenomenon and found the rate of decay of NR activity increased as the leaf age increased in all species studied. NR activity was found to be relatively stable in certain maize genotypes but extremely unstable in others while in oats, wheat, barley, rye and tobacco no genotypes with stable NR were found. These authors estimated that nitrate-reducing capacity may have been underestimated by as much as 15-fold in some cases which is of great significance when one considers that NR activity has been used as an index in selecting desirable stocks in plant breeding programs (Hageman *et al*, 1963) and for comparison with amounts of reduced nitrogen accumulated (Eilrich and Hageman, 1973).

It is easy to envisage further situations where interpretation of data is made impossible by this phenomenon. Therefore, in order to make the *in vitro* assay of NR more meaningful, attempts were made by various

workers to obtain optimum conditions for stabilisation and extraction of NR.

Many authors have reported the use of non-plant proteins such as BSA and casein for preventing the decline in stability of extractable enzyme which is characteristic of older tissue (Schrader *et al*, 1974a; Wallace, 1975b; Tischler *et al*, 1978). Although casein was able to increase stability of NR it was found that less enzyme could be extracted from the tissue in its presence (Sherrard and Dalling, 1978).

It is not known how exogenous protein brings about these effects but it has been suggested that enhancement of NR activity by BSA may be due to its ability to form hydrogen bond complexes with phenolic compounds so removing them as inhibitors of NR (Purvis *et al*, 1976). Evidence for this came from the observation that extraction in the presence of Dowex 1-Cl, which is known to remove phenolic enzyme inhibitors from solution (Lam and Shaw, 1970), also caused an increase in NR activity. The same authors also noted that delayed addition of BSA was equally effective as BSA present from the time of extraction. This lead them to suggest that the major effect of BSA was to make NR more active rather than increase the amount extracted or increase stability, a situation which is consistent with removal of phenolic inhibitors.

Schrader *et al* (1974a) found that retarded decay of NR activity in the presence of exogenous protein was not

due to an osmotic effect during the extraction procedure.

It has also been suggested that BSA may protect NR from physical denaturation or dissociation of the complex by maintaining a high protein environment (Schrader *et al*, 1974a; Sherrard and Dalling, 1978). This is supported by the observation that dilution of extracts from cotton cotyledons resulted in a more labile NR (Tischler *et al*, 1978).

Alternatively it has been suggested that NR may bind to BSA thus preventing inhibition or denaturation (Sherrard and Dalling, 1978) since it has been reported that NR binds to ruptured cell organelles, but only in the absence of BSA (Dalling *et al*, 1972).

BSA has also been postulated to protect NR activity by acting as an alternate substrate for endogenous proteinases (Wallace, 1974; 1975a). Schrader *et al* (1974a) proposed that proteolytic degradation might be responsible for the exponential decay of NR activity *in vitro*. However, Purvis *et al* (1976) were unable to relate increased instability of NR to development of proteinase activity in expanding cotton cotyledons.

Although non-specific inactivation by proteinases or inhibitors from other cell compartments released on extraction cannot be ruled out as a reason for lability of NR, NR-specific inactivating systems have been actively sought to account for this phenomenon.

Two main types of NR inactivator have been discovered. The first causes reversible inactivation of NR by proteolysis while the second associates with the NR complex, usually in a reversible fashion, in such a way as to cause loss of activity.

Different types of NR inactivator have been reported from the same source tissue. In maize roots, Pan and Marsh (1972) detected a protein-like macromolecular inhibitor of NR, Aslam (1977) found heat inducible NR inhibitor(s) in the root tips while Wallace (1974) has observed a heat labile inactivating enzyme from the mature root region which was able to promote inactivation of the relatively stable NR in root tip extracts. The inactivator discovered by Wallace was also present in root tips but its activity in crude extracts was difficult to detect due to the presence of an inhibitor (Wallace, 1975b; 1978). The inactivator was also found, but to a much lesser extent, in scutella and leaves of maize and in the roots and shoots of pea seedlings (Wallace, 1975a). The factor was inhibited by casein (Wallace, 1975a) but not by pCMB or cysteine (Wallace, 1974; 1975b) and although EDTA and 1,10-phenanthroline had only minor inhibitory effect (Wallace, 1974; Shannon and Wallace, 1979) PMSF totally inhibited its activity and it was therefore considered to be a serine proteinase (Wallace, 1974). The proteinase was active on alanyl ester substrates suggesting that it has the 'tight' elastase type of active site (Solomonson *et al*, 1984 personal

communication to Wallace and Oaks, 1984). The molecular weight of the maize root proteinase has been reported as 44 000 (Wallace, 1974), 54 000 (Shannon and Wallace, 1979), 66-75 000 (Yamaya *et al*, 1980a) and 56 000 (Smith, 1983). The maize root inactivator was found to cause differential loss of NR activities (Wallace, 1973b, 1975b), the BVH-NR activity being relatively resistent to inactivation (Batt and Wallace, 1983) while the NADH-CR activity appeared to be preferentially inactivated. Although the inactivator was initially considered to be specific for NR (Wallace, 1973b) it was later found to inactivate yeast tryptophan synthase (Wallace, 1978) and to have proteolytic activity towards azocasein and haemoglobin (Shannon and Wallace, 1979).

Walls *et al* (1978) proposed the existence of two mechanisms for NR inactivation in *N. crassa* and Sorger *et al* (1978) reported the isolation and characterisation of two inactivators to account for these mechanisms. The factors differed markedly in pH optimum and sensitivity to EDTA, PMSF, cycloheximide and heat treatment. Inactivator II is similar to the inactivating enzyme described by Wallace with respect to PMSF sensitivity and molecular weight. It would appear to be regulated by nitrogen-nutritional status of the mycelia being postulated to be responsible for rapid *in vivo* decay of NR on nitrogen starvation. Neither of the inactivators were considered to be specific for NR.

An NR-inactivating factor has been isolated from 8 day old wheat seedlings. The inactivator could only be detected after partial purification as two NR-stabilising factors were also present (Sherrard *et al*, 1979a; 1979b). The inactivator was heat-labile and had a MW of 37 500. It was sensitive to Fe^{2+} chelators, iodoacetamide, trypsin inhibitors TLCK and α N-benzoyl-L-arginine, BSA and casein but not to PMSF. The inactivator was considered likely to be a proteinase despite the authors inability to detect hydrolytic activity towards casein, haemoglobin and several artificial substrates. This factor was more active towards NADH-NR activity than $FMNH_2$-NR or MVH-NR activities and in contrast to the maize enzyme, the NADH-dehydrogenase activity was least affected by it (Sherrard *et al*, 1979b).

Kadam *et al* (1974; 1975) have detected an inhibitor of NR from roots of rice plants which appears to be a peroxidase since the effect of the inhibitor and of horse-radish peroxidase on NR are almost identical. This peroxidase-like factor has a MW of approximately 40 000 and inhibits NADH- and $FMNH_2$-NR activities but not MVH-NR activity and is not affected by the presence of casein.

A much larger, heat labile, NR inactivating factor (MW 200 000) has been isolated from rice cells in suspension culture (Yamaya and Ohira, 1976; 1977; 1978a; 1978b) which had no proteolytic activity towards BSA, casein or NR (Yamaya and Ohira, 1977). It was

inhibited by 1,10-phenanthroline, EDTA and pCMB (Yamaya and Ohira, 1977) but not by BSA, casein, cysteine, nitrate or PMSF and is considered to be a NR-binding protein rather than a proteinase. The factor inhibited all NR activities except MVH-NR activity (Yamaya and Ohira, 1976; 1977; 1978a) and was not effective in the presence of NADH, suggesting that it binds only to the oxidised form of NR. NADH could not protect NR in the presence of nitrate or cytochrome *c* (Yamaya and Ohira, 1978a) but has been shown to reverse the effect of the inactivating factor (Yamaya and Ohira, 1978b). The inhibitor was thought to be specific for NR but it was able to inactivate the molybdoflavoprotein xanthine dehydrogenase (Yamaya and Ohira, 1977) which may be of significance. Leong and Shen (1982) have shown that the bound form of the inactivator can still inactivate subsequently added NR suggesting that there may be more than one active site on the inhibitor molecule.

Jolly and Tolbert (1978) have isolated a heat labile NR-inactivator from young dark-grown soybean leaves. It had a MW 31 000 with two identical subunits and did not appear to be a proteinase but rather a binding protein since its mobility changed through Sephadex G-75 in the presence of soybean leaf NR. In addition, it was inactivated in the light, a process which is thought to occur via reversible conformational change in the inhibitor. The inhibitor inactivated all NR activities, including MVH-NR but had no effect on NADH-CR activity. In contrast to the

rice cell inactivator it was therefore considered to act on the nitrate-reducing region of the NR complex. The soybean NR-inactivator is considered to be involved in regulation of light/dark changes in NR activity and thus have a definitive *in vivo* function. The *in vivo* role, if any, of other inactivators described here may not be so well defined. Possibilities regarding this topic will be investigated in the General Discussion in the light of the work to be described in this thesis.

The aims of the work described in this thesis were:

1) To determine the effects of haem deficiency on synthesis/assembly of NR and to investigate the involvement of haem in the NADH-CR activity and $FMNH_2$-NR activity of the enzyme complex.

2) To investigate the phenomenon of age-dependent *in vitro* stability of NR by determining the mode of inactivation of NR in extracts of older tissue and to assess conditions under which NR could be stabilised *in vitro*.

3) To examine the controlled proteolysis of purified NR with respect to differential loss of partial activities to determine likely sites of proteolytic attack and thus obtain information on the structure and regulation of NR. Trypsin and inactivating factors purified by others working in this laboratory from maize and barley were employed for this purpose and conditions under which NR could be protected from cleavage also determined.

4) To raise monospecific antibodies against NR in order to determine the extent to which loss of NR activity correlates with loss of NR cross-reacting material under various conditions such as growth on ammonium and also to characterise proteolytic products of NR.

5) To purify the small 40 000 NADH-CR species observed in crude extracts, especially of older tissue, in order to further characterise this species (especially with respect to haem-content) and to raise monospecific antibodies against it in order to determine the relationship between this species and NR.

MATERIALS

CHEMICALS

The following were obtained from the Sigma London Chemical Company: Agarose (I.D.); Alcohol dehydrogenase (Yeast); Azocasein; Bovine serum albumin (crystalised and lyophilised); Catalase (bovine liver); Coomassie Brilliant Blue G250; Coomassie Brilliant Blue R250; Cysteine (free base); Cytochrome *c* (horse heart, type III); Dithiothreitol; FAD (grade III); FMN (sodium salt, synthetic); Methyl viologen; Myoglobin (whale skeletal muscle, type II); NAD^+ (yeast, grade III) NADH (yeast, grade III); Nitroblue tetrazolium (grade III); ovalbumin (chicken egg, grade V); 1,10-phenanthroline (monohydrate); Phenylmethylsulphonyl fluoride; phosphorylase b (rabbit muscle); 3,3',5,5', tetramethyl benzidine; 2,3,5-tetrazolium chloride; Trypsin (bovine pancreas, type III) and Trypsin inhibitor (Egg white, type II-o).

Acrylamide (electrophoresis grade), N,N'-methylene bis-acrylamide (electrophoresis grade) and laevulinic acid were obtained from British Drug Houses Ltd.

Sephadex G200, CNBr-activated Sepharose 4B and Blue Dextran 2 000 were obtained from Pharmacia, Sweden.

Biogel A 1.5 m was obtained from Biorad Laboratories Ltd., Watford, Hertfordshire.

Szechrome NAS was obtained from Polysciences Inc., Warrington, PA 18976.

Drakeol 6VR was obtained from Pensilvania Oil Refining Co., U.S.A.

Arlacel was obtained through Serva Chemical Co., Heidelberg, W. Germany.

Freund's complete adjuvant was obtained from the Grand Island Biological Company, Grand Island, N.Y. 14072, U.S.A.

All other chemicals were of the highest grade available from the usual commercial sources.

SEEDS

Barley (*Hordeum vulgare* cv Golden Promise) seeds were obtained from William Watt and Sons, Cupar, Fife.

METHODS

SECTION I: GROWTH OF PLANTS

(a) In the Laboratory

Barley (*Hordeum vulgare* cv. Golden Promise) seeds were sown in trays containing vermiculite, watered with tap water and placed in darkness at 28°C to germinate. After 68 h, when the coleoptiles were approx 1 cm long, plants were transferred to continuous light (1 000 lux) supplied by 3 Grolux fluorescent tubes at 26°C. Plants were treated daily with a modified half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938; Table 2).

(b) In the Field

Barley seeds were sown in rows 25 cm apart with Fisons 52 Regular fertiliser at a rate of 400 kg/ha.

SECTION II: EXTRACTION AND PURIFICATION PROCEDURES

(a) Purification of Nitrate Reductase (NR)

NR was extracted and purified by a slight modification of the method developed by Small (1980).

All purification steps were carried out between 0 and 4°C.

90 h old barley shoots (approx 4 cm long) were harvested and homogenised in a chilled mortar and pestle with cold 0.05M potassium phosphate buffer pH 7.5 containing 0.1mM EDTA, 10µM FAD and 1mM cysteine (Buffer 1). An extraction ratio of 3 ml buffer to 1 g fresh weight of tissue was used.

Table 2: Composition of half-Hoagland nutrient solution

Stock solution	Composition	g/l
A	NaFeEDTA	38.44
B	KH_2PO_4	34.25
	$MgSO_4.7H_2O$	126.65
	$ZnSO_4.7H_2O$	0.0555
	$MnSO_4.H_2O$	0.3904
	$CuSO_4.5H_2O$	0.0206
	H_3BO_3	0.7250
	$Na_2MoO_4.2H_2O$	0.00622

10 ml of each stock solution were diluted to 5ℓ with distilled water and 8 g of KNO_3 added to give a final nitrate concentration of 15.8mM.

KNO_3 was omitted for the nitrate-less half-Hoagland nutrient solution.

The brei was squeezed through a double layer of muslin and the resultant filtrate centrifuged at 38 000 g in an MSE High Speed 25 Centrifuge at 4°C for 40-60 min. The supernatant was adjusted to 45% $(NH_4)_2SO_4$ saturation by the slow addition of solid $(NH_4)_2SO_4$ at 0-4°C with stirring for 15-20 min. The pH was maintained at 7.5 by by the addition of a dilute solution of ammonium hydroxide. The precipitated protein was collected by centrifugation in an MSE High Speed 25 centrifuge at 4°C and 20 000 g for 30-40 min and redissolved in Buffer I to give a final volume of 100 ml. The high molecular weight green components were removed by centrifugation for 20 min at 100 000 g (40 000 rpm) in an MSE Superspeed 65 Mkl centrifuge. The supernatant was concentrated by 0-45% $(NH_4)_2SO_4$ fractionation. Precipitated proteins were collected by centrifugation in an MSE High Speed 18 centrifuge for 20 min at 4°C and 38 000 g and redissolved in a minimum volume of Buffer I (usually approximately 20 ml).

A maximum volume of 20 ml of this sample, containing up to 800 mg protein, was applied to a Biogel A 1.5 m 100-200 mesh column (4.1 x 86 cm) pre-equilibrated with Buffer I. NR was then eluted with Buffer I at a flow rate of 5 ml/min and 15 ml fractions were collected by means of a Copey Fractomin fraction collector. The peak NR-containing fractions were pooled and concentrated by 0-45% $(NH_4)_2SO_4$ fractionation. The precipitated proteins were collected by centrifugation and were redissolved in

a minimum volume (usually approximately 3 ml) of 0.02M potassium phosphate buffer pH 7.5 containing 0.1mM EDTA, 10µM FAD and 1mM cysteine (Buffer III). The sample was usually stored at this stage in 40% glycerol at -70°C.

Prior to the final stage of the purification the sample was thawed at room temperature and glycerol removed by 0-50% $(NH_4)_2SO_4$ fractionation using an equal volume of saturated $(NH_4)_2SO_4$ solution pH 7.5. Precipitated proteins were collected by centrifugation and redissolved in 10 ml of Buffer III.

The sample was then applied to a Blue-Dextran Sepharose column (1.8 x 8.5 cm) pre-equilibrated with Buffer III. The column was washed with Buffer III until the absorbance at 280 nm was less than 0.05. Nitrate reductase was eluted with Buffer III containing 5µM NADH at a flow rate of 1-2 ml/min and 6 ml fractions were collected by means of an LKB Ultrorac fraction collector.

The peak NR-containing fractions were pooled and either used as the enzyme source in this form or concentrated against PEG 6 000 depending on the investigation.

(b) Purification of a Nitrate Reductase-Related NADH-Cytochrome c Reductase (CR) Species

The 40 000 MW NADH-CR species was extracted and purified essentially by the method developed by Small (1980).

144 h-old barley shoots (primary leaves approximately 10-12 cm long) were harvested and ground with a chilled mortar and pestle and Buffer I lacking cysteine (Buffer II). An extraction ratio of 3 ml of buffer to 1 g fresh weight of tissue was used. A little acid-washed sand facilitated the extraction of this fibrous older tissue. The brei was squeezed through a double layer of muslin and the resultant filtrate centrifuged at 38 000 g in an MSE High Speed 25 centrifuge at 4°C for 40-60 min. The supernatant was adjusted to 30% $(NH_4)_2SO_4$ saturation by the slow addition of solid $(NH_4)_2SO_4$ at 0-4°C with stirring for 15-20 min. The pH was maintained at 7.5 by the addition of a dilute solution of ammonium hydroxide. The precipitated protein was collected by centrifugation and the supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$ as previously described. Precipitated protein was collected by centrifugation and redissolved in a minimum volume of Buffer II (usually 20 ml).

A maximum volume of 20 ml of this sample containing up to 2 000 mg proteins were applied to a Biogel A 1.5m 100-200 mesh column (4.1 x 86 cm) pre-equilibrated with Buffer II. NADH-CR activity was then eluted with Buffer II at a flow rate of 5 ml/min and approximately 15 ml fractions were collected. The peak NADH-CR-containing fractions were pooled and concentrated by 0-60% $(NH_4)_2SO_4$ fractionation. The precipitated proteins were collected by centrifugation and redissolved in a minimum volume of Buffer III (usually approximately 3 ml). The sample was usually stored at this stage in 40% glycerol at -70°C.

Prior to the final stage of the purification the sample was thawed at room temperature and glycerol removed by 0-67% $(NH_4)_2SO_4$ fractionation using twice the volume of a saturated $(NH_4)_2SO_4$ solution, pH 7.5. Precipitated proteins were collected and redissolved in Buffer III (10 ml).

The sample was then applied to a Blue-Dextran Sepharose column (1.8 x 8.5 cm) pre-equilibrated with Buffer III lacking cysteine, Buffer IV. The column was washed with Buffer IV until the absorbance at 280 nm was less than 0.05, NADH-CR activity was eluted with Buffer IV containing 5µM NADH at a flow rate of 1-2 ml/min and 6 ml fractions were collected.

The peak NADH-CR-containing fractions were pooled and either used as the enzyme source in this form or

concentrated against PEG 6 000 depending on the investigation.

(c) Preparation of Crude Barley Leaf Extracts

When crude extract was required for analysis the shoot material was homogenised with cold Buffer I using a chilled mortar and pestle. The brei was either filtered through a double layer of muslin and the resultant filtrate used, or filtered through muslin and centrifuged at 38 000 g in an MSE High Speed 18 centrifuge for 20 min at 4°C and the supernatant used (cell-free extract).

SECTION III: ASSAY PROCEDURES

(a) Enzyme Assays

NADH-Nitrate Reductase (NADH-NR)

NADH-NR was assayed by the method of Wray and Filner (1970). The reaction mixture contained 0.5 ml of 0.1M potassium phosphate buffer pH 7.5, 0.1 ml of 0.1M KNO_3, 0.1 ml of 1mM NADH, enzyme sample and distilled water to a final volume of 1 ml. The reaction was started by the addition of the enzyme sample. After a suitable period of time at 25°C, the reaction was terminated by the addition of 1 ml of 1% (w/v) sulphanilamide in 3M HCl then 1 ml of 0.02% (w/v) N-1-naphthylethylene-diamine dihydrochloride (NED) (Snell and Snell, 1949). After thorough mixing the precipitated protein was sedimented in a bench-top centrifuge at top speed for 5 min.

After allowing a further 15 min for maximal colour development the absorbance at 540 nm of the supernatant was measured against a control in which the sulphanilamide was added before the enzyme sample. Absorbance values were then converted to nmol of nitrite by means of a previously established calibration plot of 0-100 nmol of potassium nitrite (Fig.3).

$FMNH_2$-Nitrate Reductase ($FMNH_2$-NR)

$FMNH_2$-NR was assayed by a modification of the method of Paneque *et al* (1965). The reaction mixture contained 0.4 ml of 0.1M potassium phosphate buffer pH 7.5, 0.3 ml of 2mM FMN, 0.1 ml of 0.1M potassium nitrate, enzyme sample and distilled water to a final volume of 0.9 ml. The reaction was started by the addition of 0.1 ml of 10 mg/ml sodium dithionite in 95mM sodium bicarbonate and gentle shaking to reduce FMN to pale yellow $FMNH_2$. After a suitable incubation time at 25°C the reaction was terminated by vortex mixing to reoxidise the $FMNH_2$ to bright yellow FMN. Controls were incubated as above but without the addition of sodium dithionite. Nitrite formed was measured as previously described for NADH-NR activity.

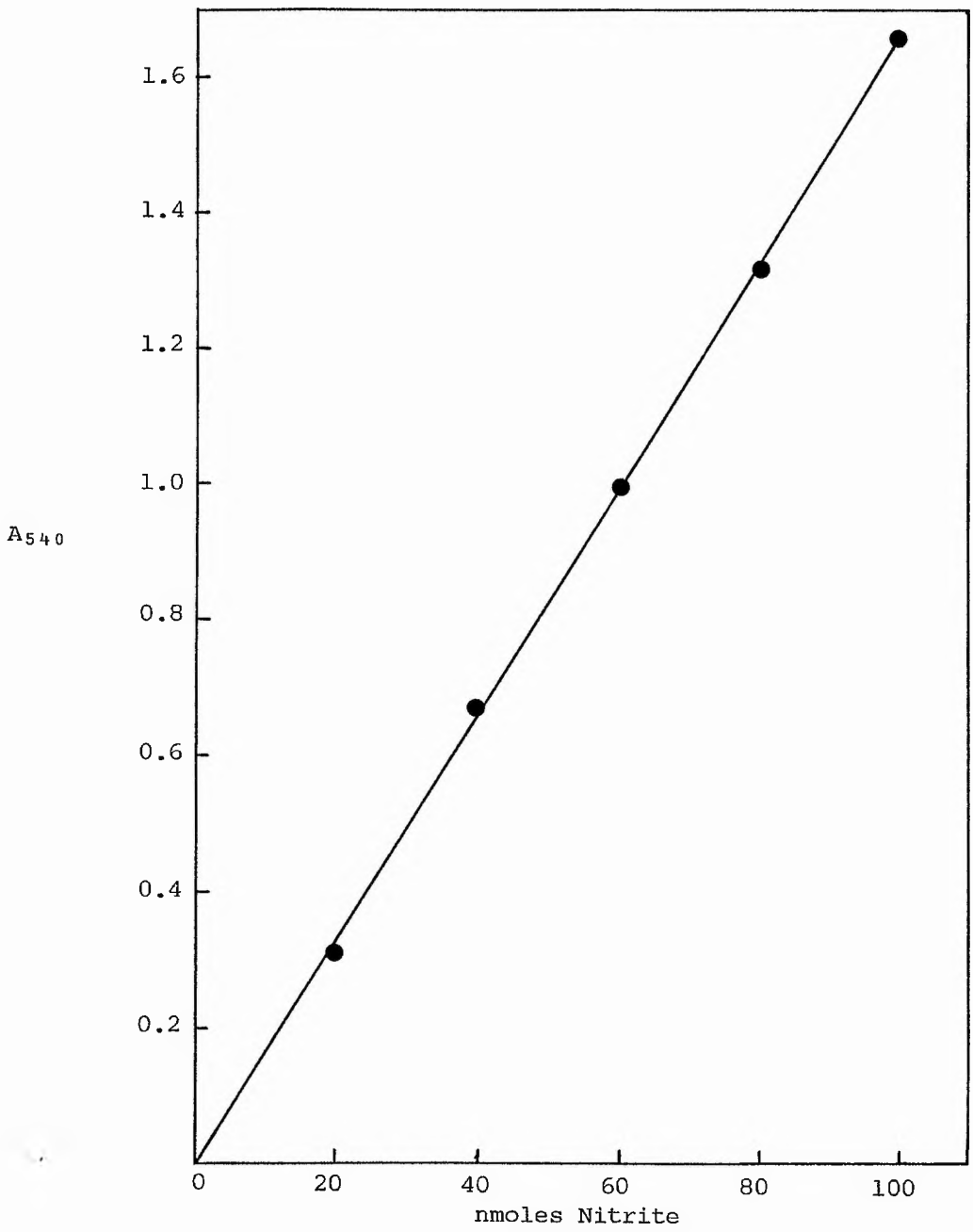
Reduced Methyl Viologen-Nitrate Reductase (MVH-NR)

The MVH-NR reaction mixture contained 0.6 ml of 0.1M potassium phosphate buffer pH 7.5, 0.1 ml of 1mM methyl viologen, 0.1 ml of 0.1M KNO_3, enzyme sample and

Fig.3

Nitrite Standard Curve

This plot shows the relationship between nitrite concentration in standard nitrite solutions and absorbance at 540 nm after addition of the Griess-Ilosvay reagents as described in the text (Methods, Section III).


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A_{540}
0
20
40
60
80
100
nmoles Nitrite

distilled water to a final volume of 0.9 ml. The reaction was started by the addition of 0.1 ml of 10 mg/ml sodium dithionite in 95mM sodium bicarbonate and gentle shaking to reduce the methyl viologen to its blue reduced form. After a suitable incubation period at 25°C the reaction was terminated by vortex mixing which reoxidised the methyl viologen to its colourless form. Controls were incubated as above but without addition of sodium dithionite. The nitrite formed was determined as previously described for NADH-NR.

To ensure the maintainance of the electron donors ($FMNH_2$ and MVH) in their reduced forms when long assay periods were required it was necessary to gently flush the incubation mixture with N_2 and seal the tubes with Nescofilm.

NADH-cytochrome c reductase (NADH-CR)

NADH-CR was assayed by the method of Wray and Filner (1970). The reaction mixture contained 0.2 ml of 0.1M potassium phosphate buffer pH 7.5, 0.02 ml of 2% (w/v) cytochrome c, 0.08 ml of 1mM NADH, enzyme sample and distilled water to a final volume of 0.4 ml. The reaction was started by the addition of enzyme sample and the reduction of cytochrome c was followed by measuring the increase in absorbance at 550 nm using a Pye Unicam SP500 Series III UV/visible spectrophotometer linked to a linear chart recorder. Change in absorbance was converted to µmol cytochrome c reduced by means of its molar extinction

coefficient (2.1×10^4 M^{-1}) using the following equation

$$\frac{\Delta A_{550}}{52.5} \times \frac{\text{Total incubation volume (ml)}}{\text{sample volume (ml)} \times t \text{ (min)}}$$

= µmol cyt c reduced/min

As cysteine interferes with NADH-CR measurement it is essential that it should be absent from the enzyme sample.

NADH-Nitroblue-tetrazolium reductase (NADH-NBT reductase)

NADH-NBT reductase was assayed by the method of Small (1980). The reaction mixture contained 0.1 ml of potassium phosphate buffer pH 7.5, 0.1 ml of 0.9 mg/ml NADH, 0.1 ml of 2 mg/ml NBT, enzyme sample and distilled water to a final volume of 0.4 ml. The reaction was started by the addition of enzyme and the increase in absorbance at 540 nm was measured by means of a recording spectrophotometer. Changes in absorbance were converted to µmol NBT reduced by means of the experimentally derived molar extinction coefficient (Small, 1980) of 5.742×10^3 using dithionite-reduced NBT. The following equation was employed

$$\frac{\Delta A_{540}}{14.355} \times \frac{\text{Total incubation volume (ml)}}{\text{Sample volume (ml)} \times t \text{ (min)}}$$

= µmol NBT reduced/min

Nitrite Reductase

Nitrite reductase was assayed by a modification of the method of Chroboezek-Kelker (1969) utilising reduced

methyl viologen as the electron donor. The reaction mixture contained 0.3 ml of 0.1M potassium phosphate buffer pH 7.5, 0.1 ml of 20mM KNO_2, 0.1 ml of 10mM methyl viologen, enzyme sample and distilled water to a final volume of 0.9 ml. The mixture was gently flushed with N_2 and the reaction started by the addition of 0.1 ml of 10 mg/ml sodium dithionite in 95mM sodium bicarbonate and the tubes sealed with Nescofilm. After a suitable incubation time at 25°C the reaction was terminated by vortex mixing until the methyl viologen was oxidised. A 0.1 ml aliquot of the reaction mixture was then pipetted into 2.9 ml of distilled water and the nitrite was determined as previously described for NADH-NR, the loss of nitrite from the reaction being a measure of nitrite reductase activity.

Catalase

Catalase was assayed by a modification of the method of Beers and Sizer (1952). 0.4 ml of 30% (v/v) hydrogen peroxide were added to 100 ml 0.1M potassium phosphate buffer pH 7.5. The reaction mixture contained 3 ml of this mixture and 30µl of enzyme sample. The reaction was started by the addition of enzyme sample and the decrease in absorbance at 240 nm was followed by means of a recording spectrophotometer.

Alcohol dehydrogenase

Alcohol dehydrogenase was assayed by a modification of the method of Vallee and Hoch (1955). The following assay mixture was prepared: 10 ml of 0.1M Tris HCl buffer pH 8.5, 10 ml of 3 mg/ml NAD^+, 5 ml of 2 mg/ml dithiothreitol and 10 ml of 1% (v/v) ethanol. The reaction was started by adding 20µl of enzyme sample to 1 ml of this mixture and the increase in absorbance at 340 nm was followed by means of a recording spectrophotometer.

Azocasein-degrading activity

Azocasein-degrading activity was measured by a modification of the method of Charney and Tomarelli (1949). A 2.5% solution of azocasein was prepared by dissolving 1.25 g of azocasein in 25 ml of 1% Na_2HCO_3 at 60°C with stirring. On cooling to room temperature the pH was adjusted to 7.5 with a solution of NaH_2PO_4 and the solution diluted to 50 ml with distilled water. 1 ml of this solution was incubated at 25°C with 1 ml of cell free extract. Controls were prepared in the same way except that 1 ml of Buffer I was added instead of extract. After 7 h (or 24 h) 2 ml of 5% TCA were added to terminate digestion. The mixture was centrifuged in a bench top centrifuge at full speed and the supernatant filtered through filter paper. 0.2 ml of 10M NaOH was then added to a 2 ml aliquot of the filtered supernatant and the absorbance at 440 nm measured against a blank after 10 min.

(b) Non-enzymic Assays

(i) Myoglobin was measured by means of its absorbance at 415 nm.

(ii) Blue Dextran 2000 was measured by means of its absorbance at 625 nm.

(c) Total Protein

Total protein was estimated by the protein-dye binding method of Bradford (1976). The protein reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol to which 100 ml of 85% (w/v) phosphoric acid was added. The solution was then diluted to 1 ℓ with distilled water and subsequently filtered on every occasion before use. 5 ml of protein reagent were added to 0.1 ml of an appropriately diluted sample and mixed thoroughly. The absorbance at 595 nm was measured after 2 min and before 1 h against a reagent blank. Absorbance was converted to protein concentration by means of a standard plot constructed from a range of BSA concentrations (0-100µg) processed with the samples (Fig.4).

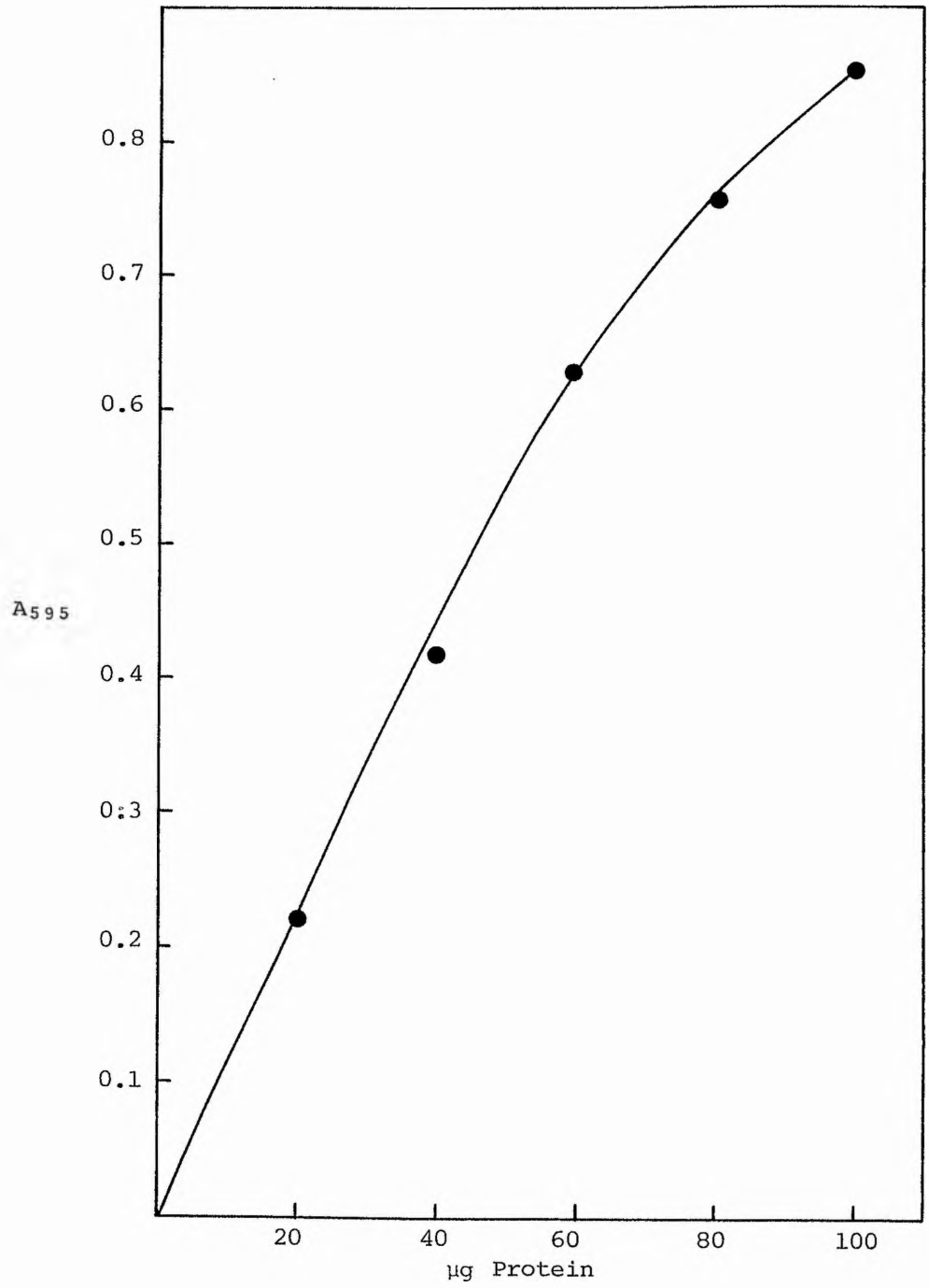
(d) Chlorophyll

Chlorophyll was measured according to the method of MacKinney, (1941). Barley shoots were ground in a mortar and pestle with 2 ml of 80% acetone and a little acid-washed sand. The pestle was washed into the mortar with 3 ml

Fig.4

Protein Standard Curve

This plot shows the relationship between protein concentration in standard solutions of BSA and absorbance at 595 nm after addition of the Bradford protein reagent as described in the text (Methods, Section III).


0.8
0.7
0.6
0.5
A595
0.4
0.3
0.2
0.1
20
40
60
80
100
µg Protein

of 80% acetone and the brei poured into a centrifuge tube. The mortar was then rinsed with 5 ml of 80% acetone and added to the tube giving a final volume of 10 ml. The tube contents were mixed by inversion then centrifuged at 20 000 g (12 000 rpm) for 10 min. The absorbance of the supernatant at 663 nm was measured and the chlorophyll concentration in µg/g FW of tissue was calculated from the equations:

$$\text{chlorophyll in } \mu g/ml = 11.9 \times A_{663}$$

$$\text{chlorophyll in 10 ml} = 119 \times A_{663} \mu g$$

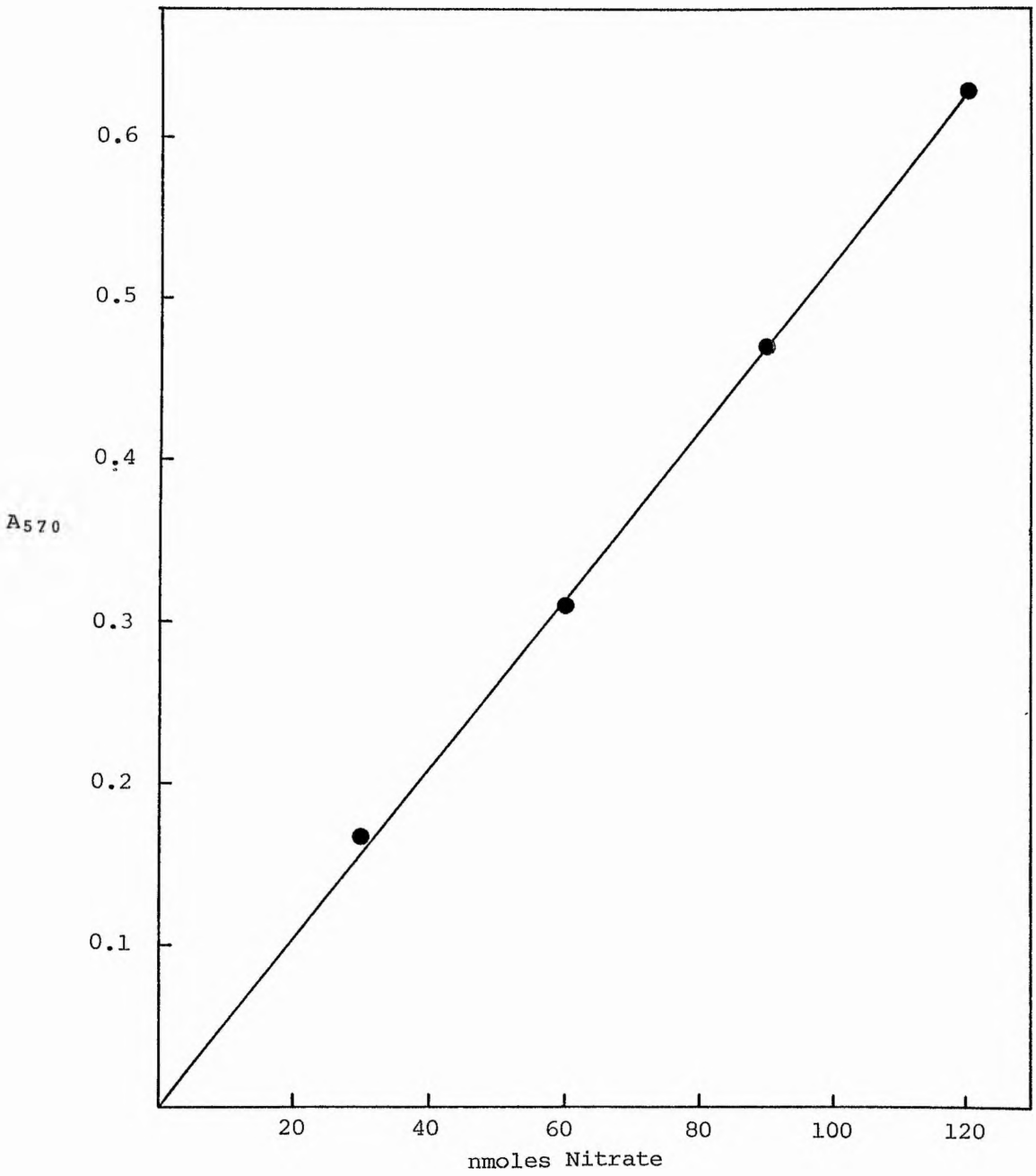
(e) Nitrate

Tissue nitrate was estimated by the Szechrome NAS method of Aviram (1975). The reagent was prepared by dissolving 5 g Szechrome NAS in a mixture of 500 ml of concentrated phosphoric acid (85-86%). The acid mixture was prepared at least 1 week prior to addition of reagent. 5 ml of the reagent were added to 0.5 ml of a suitable diluted plant extract and mixed by inversion. A violet colour was produced by the action of nitrate on the diphenylamine sulphonic acid. Absorbance at 570 nm was converted to nmol of nitrate by means of a previously established calibration plot using 0-120 nmol of KNO_3 (Fig.5).

Fig.5

Nitrate Standard Curve

This plot shows the relationship between nitrate concentration in nitrate standard solutions and absorbance at 570 nm after addition of the Szechrome NAS reagent as described in the text (Methods, Section III).


0.6
0.5
0.4
A_{570}
0.3
0.2
0.1
20
40
60
80
100
120
nmoles Nitrate

SECTION IV: ANALYTICAL PROCEDURES

(a) Sucrose Density Gradient Centrifugation

18 ml, 2-18% linear gradients were constructed by carefully layering 2 ml each of 18, 16, 14, 12, 10, 8, 6, 4 then 2% sucrose solutions into EDTA-cleaned 25 ml polypropylene centrifuge tubes (MSE-34411-138). The sucrose solutions were prepared by diluting a stock solution of 20% (w/v) sucrose in 0.1M potassium phosphate buffer pH 7.5 containing 1mM EDTA and 10μM FAD with a similar buffer lacking sucrose. The gradients were allowed to equilibrate at 4°C for 18-20 h before use.

The enzyme samples to be analysed were mixed with aliquots of the reference proteins catalase, alcohol dehydrogenase and myoglobin (S values 11.3, 7.4 and 2.04 respectively). 0.4 ml of this mixture was then layered onto each gradient. The gradients were centrifuged using a 3 x 25 ml swing out rotor in an MSE Superspeed 65 Preparative Ultracentrifuge Mark I or Mark II at 4°C and 129 500 g (30 000 rpm) for 24 h.

Gradients were fractionated from the bottom by means of an MSE tube piercer. 15 drop fractions were collected resulting in an average of 41 fractions per gradient. The refractive indicies of fractions were measured by means of a Bellingham and Stanley Abbé-type refractometer to assess gradient linearity.

The distributions of catalase, alcohol dehydrogenase and myoglobin were determined and plotted against fraction

number. The peak fraction number for each reference protein was then plotted against its known sedimentation coefficient. Using the linear calibration plot so produced, the sedimentation coefficient of any component in the sample could be estimated from its peak fraction number.

(b) Sephadex G200 Gel Filtration

Samples for analysis were mixed with aliquots of the reference proteins catalase, alcohol dehydrogenase and myoglobin having Stokes radii of 5.2 nm, 4.6 nm and 2.0 nm respectively. 1 ml of this mixture was applied to a Sephadex G200 column (2 x 52 cm) pre-equilibrated with Buffer II. The column was then eluted with Buffer II at a flow rate of approximately 12 ml/h. 2 ml fractions were collected using an LKB Ultrorac fraction collector and all column operations were carried out below 4°C.

The elution volumes of the reference proteins were related to their Stokes radius by means of the correlation of Porath (1963):

$$K_d = \frac{V_e - V_o}{V_t - V_g - V_o}$$

where K_d is the distribution coefficient; V_e is the elution volume; V_o is the void volume (determined separately using Blue Dextran 2000 since it was known to bind to NR); V_t is the total volume of the column and V_g is the volume occupied by the gel particles and thereby not accessible to solvent. (V_g = B x d where B is the

bed volume per g of dry Sephadex G200, 30 ml and d is the density of dry Sephadex G200, 1.65 cm^3/g). A plot of $K_d^{\frac{1}{3}}$ against Stokes radius of the reference proteins yields a straight line from which the Stokes radius of unknown protein species could be determined.

Calculation of Molecular Weight from Stokes Radius and Sedimentation Coefficient

Molecular weights were calculated using the equation described by Siegel and Monty (1966):-

$$M = \frac{6\pi\eta N a S}{(1 - \bar{\nu}p)}$$

where M is the molecular weight; η is the viscosity of the medium (assumed to be 1); N is Avagadro's number; a is the Stokes radius in metres; S is the sedimentation coefficient in sec^{-1}; $\bar{\nu}$ is the partial specific volume (assumed to be 0.725 ml/g) and p is the density of the medium (assumed to be 1).

SECTION V: ELECTROPHORETIC TECHNIQUES

(a) Polyacrylamide Gel Electrophoresis Under Non-denaturing Conditions in 5% Polyacrylamide Gels

20 ml of a solution containing 5% (w/v) acrylamide, 0.4% (w/v) methylene bis acrylamide and 0.25% (v/v) TEMED in 0.2M Tris HCl pH 8.5 was deaerated then 5 mg of ammonium persulphate added. The solution was pipetted into glass running tubes (75 mm x 5 mm) and overlayed with distilled water to exclude air and yield a flat gel surface.

After the gels had polymerised they were placed in a Shandon apparatus for disc gel electrophoresis and the reservoirs filled with 0.08M Tris HCl buffer pH 8.5. The gels were pre-equilibrated at 1-2mA/gel at 4°C for 20 min before application of the samples prepared as follows.

1 drop of 0.05% (w/v) bromophenol blue and 1 drop of glycerol were added to 1 ml of Blue Dextran Sepharose purified enzyme preparation which had been concentrated by dialysis against PEG 6 000. 50µl of this mixture (containing approximately 5-10µg of protein) were applied to each gel.

Electrophoresis was carried out at 4°C and 2.5-3mA/gel until the tracking dye had reached within 1 cm of the bottom of the tube. Gels were then removed from the tubes and stained for protein and enzyme activities as follows:

Protein

Gels were stained for 30-60 min in a solution of methanol, distilled water and glacial acetic acid (227 : 227 : 46) containing 1.25 g of Coomassie Brilliant Blue R250. The gels were then destained with a solution of methanol, glacial acetic acid and distilled water (250 : 75 : 675).

Reduced Methyl Viologen - NR Activity

This stain is a modification of the method used by Hucklesby and Hageman (1973) for nitrite reductase. The

staining solution per gel contained 3.5 ml of 0.1M potassium phosphate buffer, pH 7.5, 0.5 ml of 0.1M potassium nitrate, 0.5 ml of 5mM methyl viologen and 0.25 ml of 10 mg/ml sodium dithionite in 95mM sodium bicarbonate. Immediately after the appearance of an achromic band at the site of enzyme activity against blue background staining, the gels were transferred to 2.5% (w/v) triphenyltetrazolium chloride. This makes the stain permanent by reacting with the reduced viologen to produce a red formazan derivative while leaving the band of reduced methyl viologen - NR activity colourless.

NBT-Reductase Activity

Each gel was stained in 0.1M potassium phosphate buffer pH 7.5 containing 1 mg NADH and 1 mg NBT. NBT-reductase activity was detected by the appearance of purple bands which were permanent provided darkness was maintained.

Haem

Haem staining was performed by the method of Thomas *et al* (1976). Gels were incubated at room temperature in the dark for 1-2 h in 6.3mM 3,3',5,5'-tetramethyl benzidine (TMBZ) dissolved in methanol (3 parts) and 0.25M sodium acetate buffer, pH 5.0 (7 parts). 30% (v/v) hydrogen peroxide was then added to give a final concentration of 30mM. The pale blue stain became visible against a clear background within minutes when maintained in the dark.

(b) SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to a modified method of Laemmli (1970) using the following stock solutions:

(A) Acrylamide solution

30% acrylamide, 0.8% methylene-bis-acrylamide in distilled water.

(B) Separation gel buffer

1.5M Tris HCl pH 8.8.

(C) Stacking gel buffer

0.5M Tris HCl pH 8.0.

(D) Reservoir buffer

0.05M Tris/0.384M Glycine pH 8.3 containing 0.1% SDS

For 20 ml of 7.5% separating gel 5 ml (A), 5 ml (B), 9.8 ml H_2O, 0.2 ml 10% SDS and 0.8 ml 10% ammonium persulphate (made up fresh) were combined and degassed on ice. 15µl TEMED were then added, mixed and the solution pipetted into glass tubes (100 mm x 5 mm) allowing adequate space for the stacking gel. The gels were overlayed with distilled water and allowed to set. For 10 ml of 3% stacking gel 1 ml (A), 2.5 ml (C), 6.4 ml H_2O, 0.1 ml 10% SDS and 0.06 ml 10% ammonium persulphate (freshly prepared) were combined and degassed on ice. 10µl TEMED were then added, mixed and after removal of the water overlay the stacking gel solution was layered on top of the separation gel. The gels were overlayed with

water and allowed to set. After the stacking gels had polymerised the water overlay was removed and the gels placed in a Shandon apparatus for disc gel electrophoresis and the reservoirs filled with electrode buffer (D).

Solid SDS and 2-mercaptoethanol were added to the samples to give a final concentration of 2% and 1% respectively. (This was found to be necessary due to the small samples available for analysis) They were then boiled for 5 min and allowed to cool before subjecting to electrophoresis. The standard protein solution containing phosphorylase b (92 000 MW), BSA (68 000 MW), ovalbumin (45 000 MW) and myoglobin (17 200 MW) in electrode buffer (1 mg of each per ml) was treated in exactly the same way as the samples. 1 drop of 0.05% (w/v) bromophenol blue and 1 drop of glycerol were added to 1 ml of samples and standard solution and 50µl aliquots were applied to separate gels.

Electrophoresis was carried out at 0.5mA/gel until the sample had fully entered the separation gel when the current was increased to 2.5mA/gel until the tracking dye had reached within 1 cm of the bottom of the gel.

Gels were removed from the tubes, the dye front marked by piercing through the mid-point with a needle dipped in indian ink and then stained using Coomassie Brilliant Blue Staining solution (as described previously). After 1 h the gels were exhaustively destained in the destaining solution previously described then scanned using a Fison's Vitatron scanning densitometer.

The relative mobilites of the protein bands were calculated from the formula:

$$R_f = \frac{\text{distance of protein migration}}{\text{distance of dye migration}}$$

A semilogarithmic plot of mobility of the standard proteins against their known molecular weights was constructed from which the molecular weights of proteins in the enzyme samples were estimated.

SECTION VI: PREPARATION OF BLUE DEXTRAN SEPHAROSE

The procedure for the coupling of Blue Dextran 2 000 to Sepharose 4B was essentially that described by Sherrard and Dalling (1979). 3 g of commercially prepared CNBr-activated Sepharose 4B were swollen in ice-cold 1mM HCl overnight then washed on a sinter with excess 1mM HCl to remove preservatives. 10 ml swollen gel was then transferred to 20 ml of a solution containing 0.4 g of Blue Dextran 2 000 in 0.4M Na_2CO_3 pH 8.0. The suspension was gently mixed in an end-over-end tumbler for 18 h at 4°C. The gel was then washed on a sinter with distilled water to remove excess dye and suspended in 0.1M Tris HCl buffer pH 8.0 for 2 h at 4°C to block unreacted active sites. The gel was then washed successively with 0.1M sodium citrate buffer pH 4.0 and 0.1M borate buffer pH 8.0 until A_{280} of the washings was negligible.

The extent of coupling of Blue Dextran 2 000 to CNBr-activated Sepharose 4B was determined using the following equation:

$$\text{Amount of dye bound/ml of gel} = \frac{400 \times \frac{\text{Final } A_{600}}{\text{Initial } A_{600}}}{\text{vol of swollen gel}}$$

where initial A_{600} is the absorbance at 600 nm of a solution of 0.4 g of Blue Dextran dissolved in 0.1M Na_2CO_3 pH 8.0 made up to 1 ℓ with distilled water and final A_{600} is the absorbance of the pooled washings from the coupling procedure made up to 1 ℓ with distilled water.

Using this procedure 10-12 mg of Blue Dextran 2 000 were coupled to each ml of swollen gel which compares well with the value of 13 mg/ml reported by Sherrard and Dalling (1979).

After use, the Blue Dextran Sepharose was cleaned by eluting absorbed proteins with 3M KCl and re-equilibrating in running buffer.

SECTION VII: PRODUCTION OF ANTISERA

(a) Production of Antisera Against Barley NR

NR was purified by the procedure outlined in Methods Section II. The peak NR-containing fractions from Blue Dextran Sepharose chromatography were pooled and concentrated (10-fold) against PEG 6 000 at 4°C.

Antibodies were raised in a female Dutch rabbit by subcutaneous injection of 2 ml of this purified NR preparation (containing approximately 100µg of protein) emulsified in an equal volume of Freund's complete adjuvant. 25 days later, an additional 1 ml of a similarly produced preparation of purified NR (containing approximately 20µg of protein) was emulsified with an equal volume of a 9:1 mixture of Drekeol 6VR oil : Arlacel emulsifier, then re-emulsified with an equal volume of 2% Tween 80 in 0.9% saline and injected into the rabbit as previously described.

After 18 days and at various time intervals after the booster injection, 24 ml blood samples were collected from the ear vein of the rabbit. The whole blood was allowed to clot at room temperature for at least 1 h then separated by centrifugation at top speed in a bench-top centrifuge for 10 min. The serum was then stored in aliquots at - 70°C until required.

Pre-immune serum was prepared before the immunisation scheme commenced.

(b) Production of Antisera Against an NR-Related NADH-CR Species

Antisera against Blue Dextran Sepharose purified NR-related small NADH-CR species was raised using an identical protocol to that described above for purified NR.

SECTION VIII: IMMUNOLOGICAL METHODS

(a) Ouchterlony Double Diffusion

Double diffusion was carried out using specially constructed diffusion plates layered with 1% agarose in 0.039M Tris HCl buffer pH 8.6 containing 0.005M H_3PO_4 and 0.05M NaCl in which rosettes were formed using a 4 mm cutter (well volume 22µl).

20µl aliquots of 0 to 10-fold dilutions, with Buffer I, of cell-free extract of nitrate grown 90 h old plants were applied to the outer wells and 20µl of crude anti-NR serum, 20µl of crude antiserum raised against the small NR-related NADH-CR species or 20µl of pre-immune serum applied to the inner well. In addition 20µl aliquots of 0 to 10-fold dilutions, with 0.9% saline, of anti-NR serum, anti-small NADH-CR species serum or pre-immune serum were applied to the outer wells and 20µl of cell free extract applied to the inner well.

After the plates had been incubated overnight at 27°C in a moist environment they were washed in 1% NaCl (with one change) for 1 day, followed by washing in distilled water for 8 h. The plates were pressed with filter paper to dryness then stained for 30 min in a solution of methanol, distilled water and glacial acetic acid (227 : 227 : 46) containing 1.25 g of Coomassie Brilliant Blue R250. This was followed by brief destaining with a solution of methanol, glacial acetic acid and distilled water (250 : 75 : 675).

(b) Protection of Inhibition Assay for the Estimation of Cross Reacting Material (CRM)

A standard curve of inhibition of a cell-free extract of 90 h old nitrate grown plants (4 ml Buffer I/g FW tissue) (control NR protein preparation) by antisera was prepared on each occasion in the following way. Various amounts of anti-NR serum, made up to a final volume of 50µl with Buffer I, were mixed with 250µl Buffer I in a 1.5 ml Eppendorf tube and maintained at 4°C for 45 min. 250µl of control NR protein were then added and the mixtures maintained at 4°C for a further 45 min. The mixtures were then centrifuged in an Eppendorf bench top centrifuge at full speed for 10 min after which time the supernatants were assayed for NR activities. The activities obtained in the absence of added antiserum served as a control. That volume of antiserum which resulted in 50% inactivation of NR activity was then calculated.

To estimate the amount of CRM in cell-free extracts of interest an identical procedure was used except that various amounts of antiserum, in a final volume of 50µl with Buffer I, were first reacted with 250µl of the extract (4 ml Buffer I/g FW tissue) instead of 250µl of Buffer I, the same source of control NR protein being used throughout. That volume of antiserum which was required to inactivate 50% of the NR activity in the mixture was then calculated. Any increase in amount of antiserum required to inactivate 50% of NR activity over

that required in the standard mixtures was attributed to the presence of CRM in the crude extract included in the assay which was sparing the control NR protein from inhibition.

Use of Laevulinic Acid in the Investigation of the Role of Haem in Formation of Nitrate Reductase Activities

Higher plant NR possesses as prosthetic groups FAD^+, haem (cyt b_{557}) and molybdenum. The prosthetic group involvement in the expression of the partial activities of NR has been discussed in detail in the Introduction to this thesis. It is generally accepted that molybdenum is not required for synthesis of the NR complex as it appears that a molybdenum-free apoenzyme (with the same S-value as the active holoenzyme) may be synthesised in the absence of molybdenum which possesses NADH-CR activity but is unable to reduce nitrate with NADH or reduced dyes. In contrast to the situation with molybdenum, the role of haem in the expression of partial activities is less well understood. There is dispute as to whether cyt b_{557} is involved in NADH-CR activity (Hewitt, 1975; Notton *et al*, 1977; Maldonado *et al*, 1978; Fido *et al*, 1979) and there is doubt as to whether haem is required for $FMNH_2$-NR activity. In addition, the involvement of haem in the synthesis and/or assembly of the NR complex is not known. Haem can regulate the translation of proteins and the activity of enzymes in animal cells (Granick *et al*, 1975) and it has been postulated that it may have a similar role in plant cells (Castelfranco and Jones, 1975).

It has been shown that cut shoots of higher plants will take up laevulinic acid, a competitive inhibitor of the enzyme 5-aminolaevulinic acid dehydratase (Nandi and Shemin, 1968). Laevulinic acid inhibits the conversion

RESULTS

CHAPTER 1

STUDY ON THE USE OF LAEVULINIC ACID TO INVESTIGATE THE ASSEMBLY AND PARTIAL ACTIVITIES OF BARLEY NITRATE REDUCTASE

of δ-aminolaevulinic acid to porphobilinogen and tetrapyrroles (chlorophyll and haems). Laevulinic acid strongly inhibits chlorophyll synthesis and induction of NR in greening barley leaves (Nasrulhaq-Boyce and Jones, 1977). It is likely that competition for a very limited pool of protoporphyrin between chlorophyll synthesis and haem protein synthesis leads to a reduced ability to synthesise the b_{557} component of NR when the induction stimulus is presented.

By studying the effect of laevulinic acid on the induction of partial activities of NR and on the NR-related NADH-CR species in leaf extracts it was hoped to gain a greater understanding of the role of haem in formation of NR activities and thus extend the work of Nasrulhaq-Boyce and Jones (1977).

Effect of Varying Concentrations of Laevulinic Acid on Chlorophyll Synthesis and Induction of NADH-NR in Greening Barley Shoots

The first aim of this study was to determine a suitable concentration of laevulinic acid which would cause maximum inhibition of chlorophyll synthesis and NR induction without detrimental effects to the plants. The method employed was essentially that of Nasrulhaq-Boyce and Jones (1977).

Barley seedlings were grown in the dark at 28°C for 6 days and watered daily with half-Hoagland nutrient solution lacking nitrate. The shoots were then cut under

water and placed in beakers containing 100mM KNO_3 and 50mM KH_2PO_4 pH 7.0 and various concentrations of laevulinic acid. A constant number of plants per beaker was used. After preincubation in the dark for 4 h the cut shoots were illuminated for 24 h during which time the chlorophyll and NADH-NR levels were monitored.

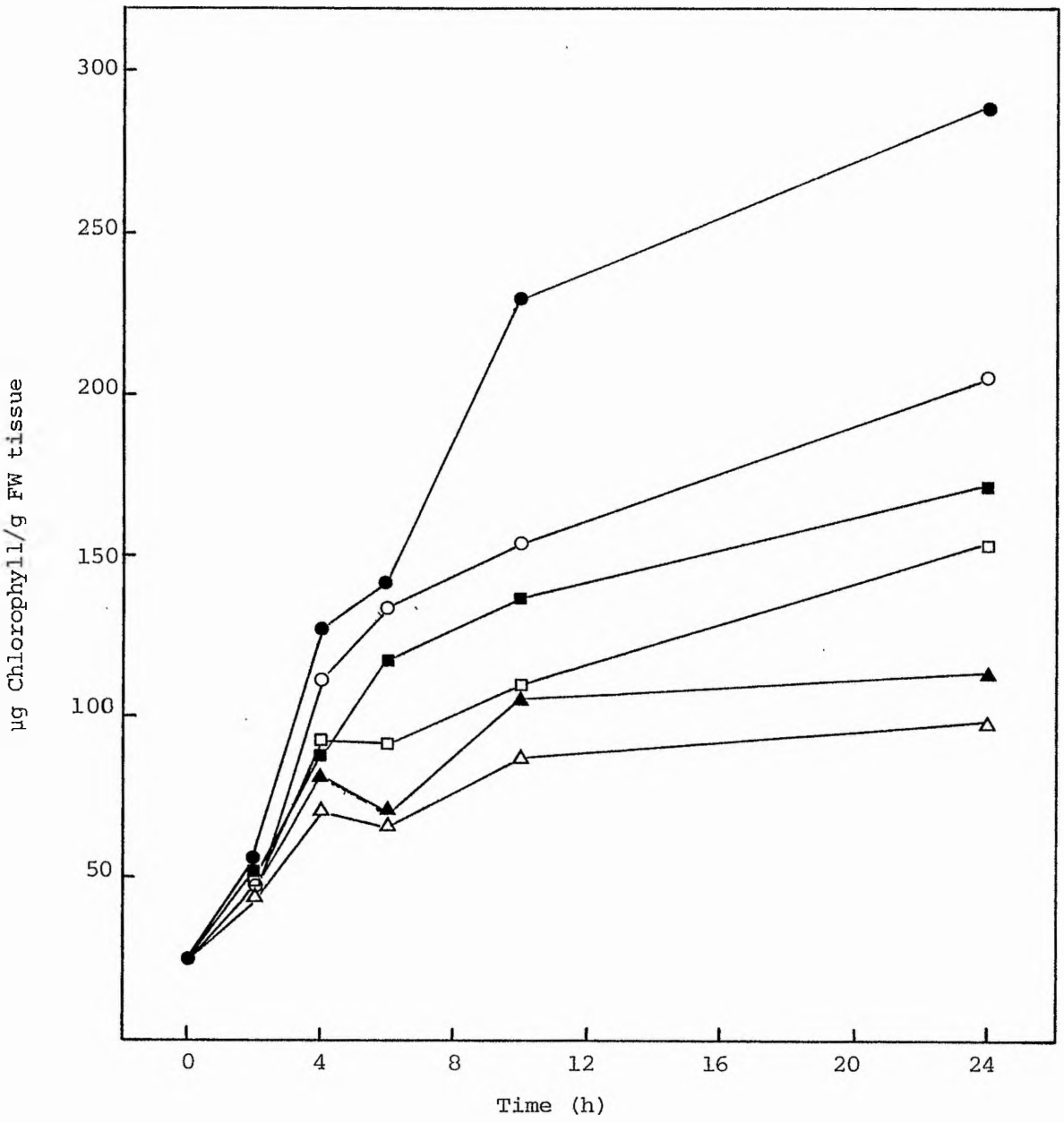
An inhibition of chlorophyll synthesis of approximately 70% was produced by 50mM laevulinic acid after 24 h (Fig.6). However, severe wilting was observed in the 40mM and 50mM laevulinic acid-treated shoots. Levels of NR in cell free extracts of the shoots were negligible, even in the control treatment, making detection of inhibition by laevulinic acid impossible (data not shown). Alternative incubation schemes were investigated to attempt to improve levels of NR activity, namely addition of solutions containing laevulinic acid to plants *in vivo* and the floating leaf segment approach employed by Beale and Castelfranco (1974) but neither proved successful (data not shown).

All parameters which departed from the routine protocol for growth of barley plants in this laboratory were investigated and it was discovered that plant age was the most important parameter to affect NR activity in shoot extracts (data not shown). Until this juncture, plant tissue of the age used (5 day old) had not been used in this laboratory since high levels of NR activity were found in 90 h old leaf tissue which was routinely used for purification of NR. The implications of this

Fig.6

Chlorophyll Levels in Cut Shoots of Greening Etiolated Barley in Varying Concentrations of Laevulinic Acid

Barley seedlings were grown in the dark at 28 °C for 6 days and watered daily with half-Hoagland nutrient solution lacking nitrate. The shoots were cut under water and placed in beakers (20/beaker) containing 100mM KNO_3 and 50mM KH_2PO_4, pH 7.0 containing 0 (●), 10 (○), 20 (■), 30 (□), 40 (▲) and 50mM (△) laevulinic acid. The shoots were pre-incubated in the dark for 4 h then illuminated for 24 h. At the times indicated shoots were removed from the beakers, rinsed with distilled water, blotted dry and weighed. The shoots were extracted in 80% acetone and chlorophyll was estimated by the method MacKinney (1941) described in the Methods, Section III.


300
250
200
150
100
50
µg Chlorophyll/g FW tissue
0
4
8
12
16
20
24
Time (h)

finding will be investigated in subsequent sections of this thesis.

Cut shoots from much younger seedling (3 day old) were therefore used for further inhibition studies. The shorter shoots made processing much more difficult and time consuming. In addition it was found that coleoptiles had to be removed if the leaves were to expand and allow maximal induction of NR activity. 30mM laevulinic acid was chosen as the working inhibitor concentration.

Effect of Laevulinic Acid on the Partial Enzyme Activities of NR and Tissue Levels of Nitrate and Chlorophyll in Greening Etiolated Barley

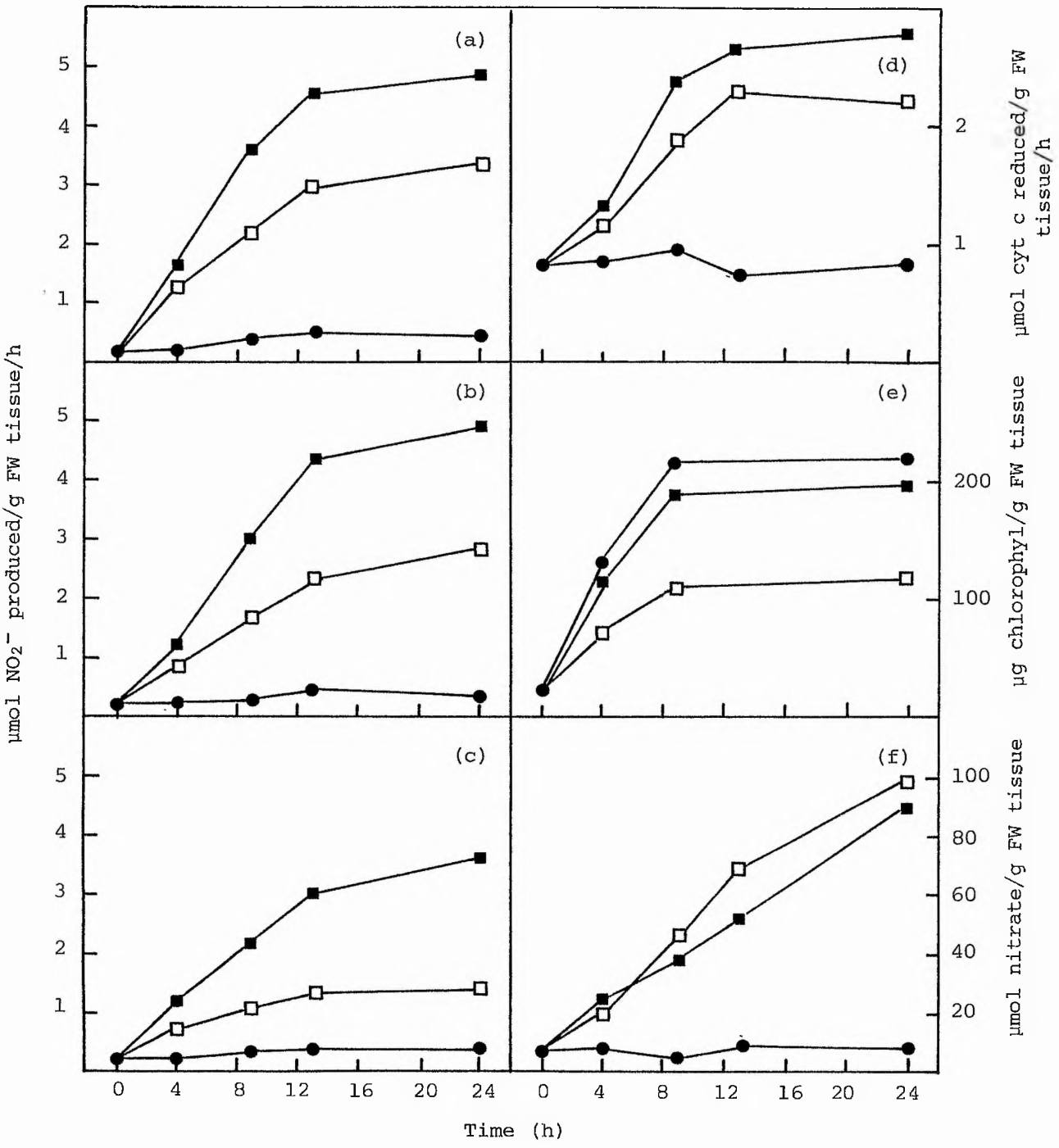
Barley seedlings were grown in the dark for 3 days and watered daily with half-Hoagland nutrient solution lacking nitrate. Shoots were cut, placed in appropriate solutions and a time course of inhibition was carried out as detailed in the legend to Fig.7.

The high level of NADH-CR activity in the extracts of cut shoots which had not received nitrate (Fig.7d) is presumably due to constitutive NADH-CR species and remained constant over the time course of the experiment. Taking this into account there was 74% of the "nitrate induced" NADH-CR activity in the laevulinic acid treatment compared to that in the control after 24 h. Similarly there was 65% of NADH-NR, 56% of $FMNH_2$-NR and 31% of MVH-NR.

Fig.7

Effect of Laevulinic Acid on the Partial Activities of NR, Nitrate and Chlorophyll Levels in the Cut Shoots of Greening Etiolated Barley

Barley seedlings were grown in the dark at 28°C for 3 days and watered daily with half-Hoagland nutrient solution lacking nitrate. The shoots were cut under water, the coleoptiles carefully removed, and placed into beakers (20/beaker) containing 100mM KNO_3 and 50mM KH_2PO_4, pH 7.0 with no additions (■) or 30mM laevulinic acid (□). A control treatment containing no nitrate or laevulinic acid was also prepared (●). The shoots were pre-incubated in the dark for 4 h then illuminated for 24 h. At the times indicated the shoots were removed from the beakers, rinsed with distilled water, blotted dry and weighed. Shoots were extracted in Buffer II (3 ml buffer/g FW tissue), filtered through muslin and centrifuged at 38 000 g for 20 min. NADH- (a), $FMNH_2$- (b), MVH-NR (c) and NADH-CR (d) activities and nitrate (f) levels were estimated for the cell-free extracts. Shoots were also extracted for the estimation of chlorophyll (e).


(a)
(b)
(c)
(d)
(e)
(f)
µmol NO_2^- produced/g FW tissue/h
µmol cyt c reduced/g FW tissue/h
µg chlorophyl/g FW tissue
µmol nitrate/g FW tissue
Time (h)
0
4
8
12
16
20
24

It would appear that induction of all the partial activities of NR are inhibited by laevulinic acid. MVH-NR activities in extracts of both laevulinic acid - treated and untreated plants were, however, consistently lower than the other partial activities of NR. This is likely to be due to the fact that nitrite reductase can also use MVH as an electron donor.

Since all of the partial activities are affected by the presence of laevulinic acid this suggests that the production of a functional NR complex is impaired when availability of haem is limited. This is reflected in the higher nitrate levels in the laevulinic acid treated tissue (Fig.7f) which is what one might expect in a situation with deficient nitrate reduction in which nitrate is likely to accumulate.

The pH of the extracts was measured to determine whether the presence of laevulinic acid in the tissue altered the buffering capacity of the extraction medium and thus indirectly affected NR activity (data not shown). This was found not to be the case since the pH of both minus and plus laevulinic acid treatment extracts were similar throughout the time course, dropping from approximately 7.35 to 7.0 over the 24 h period.

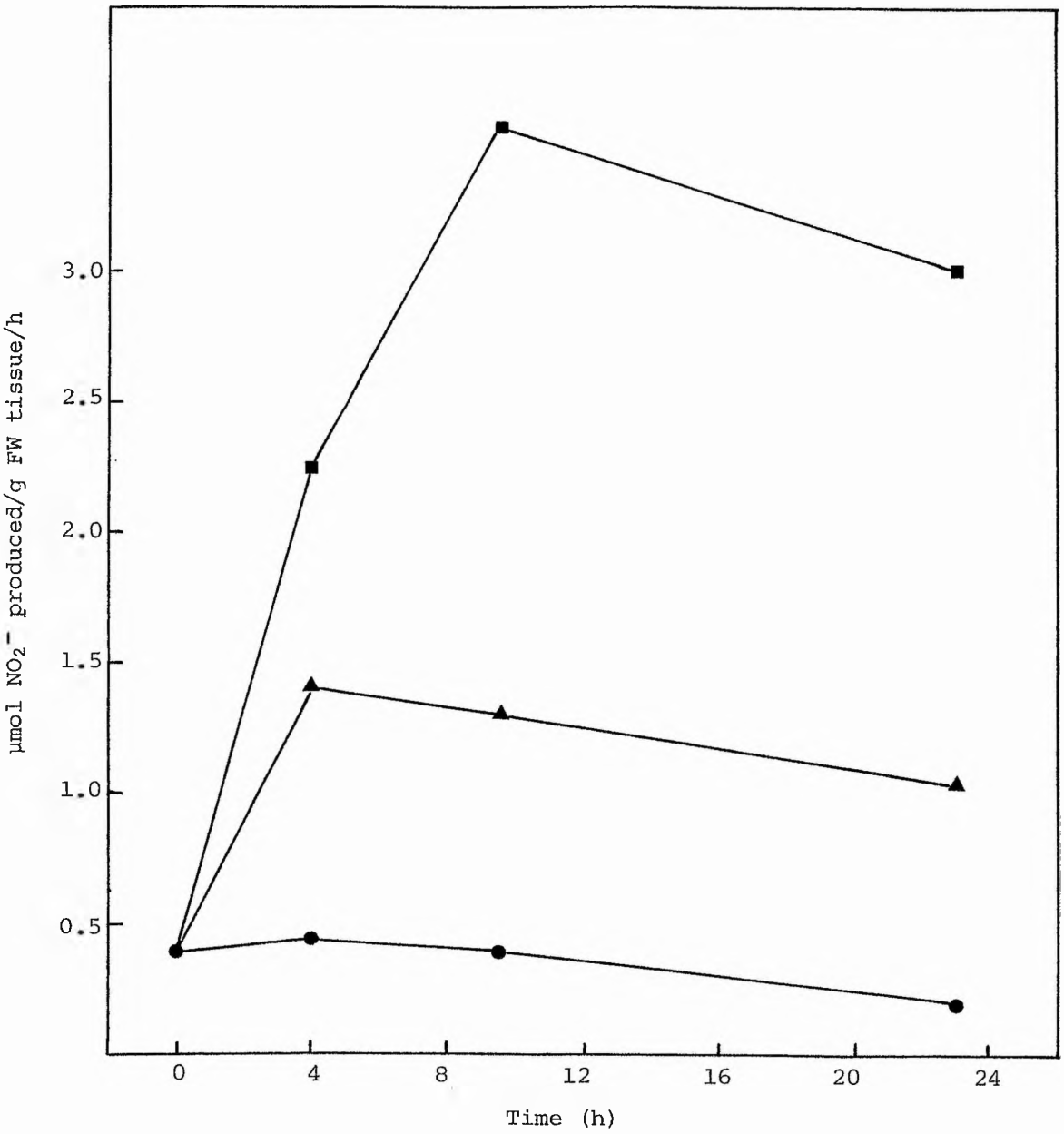
Effect of Laevulinic Acid on the Induction of NR in Fully Greened Barley

Laevulinic acid clearly inhibits haem synthesis as reflected by its effect on chlorophyll synthesis and NR

Fig.8

Effect of Laevulinic Acid on the Induction of NADH-NR Activity in the Cut Shoots of Fully Greened Barley

Barley seedlings were grown in the dark at 28°C for 3 days, illuminated for 1 day and watered daily with half-Hoagland nutrient solution lacking nitrate. The shoots were cut under water and placed in beakers (20/beaker) containing 100mM KNO_3 and 50mM KH_2PO_4, pH 7.0 with no additions (■) or 30mM laevulinic acid (▲). A control treatment containing no nitrate or laevulinic acid was also prepared (●). The shoots were illuminated for 23 h and at the times indicated shoots were removed from the beakers, rinsed with distilled water, blotted dry and weighed. Shoots were extracted in Buffer I (3 ml buffer/g FW tissue), filtered through muslin and centrifuged at 38 000 g for 20 min. The cell free extracts were then assayed for NADH-NR activity.


µmol NO_2^- produced/g FW tissue/h
3.0
2.5
2.0
1.5
1.0
0.5
0
4
8
12
16
20
24
Time (h)

activity in greening etiolated barley. However, Nasrulhaq-Boyce and Jones (1977) were also able to show that laevulinic acid inhibits NR in fully greened tissue in which one would expect there to be a readily available pool of haem for NR synthesis or assembly.

Barley seedlings were therefore grown in the dark at 28°C for 3 days, illuminated for 1 day and watered daily with half-Hoagland nutrient solution lacking nitrate. Shoots were then cut, placed in solutions as before and a time course of inhibition of NADH-NR activity by laevulinic acid in fully greened barley was carried out as detailed in the legend to Fig.8.

The results presented in Fig.8 confirm the findings of Nasrulhaq-Boyce and Jones (1977) that the nitrate-dependent induction of NR is inhibited by laevulinic acid as well as the light-dependent induction of NR described previously. This shows that laevulinic acid is likely to be affecting NR directly and not through some inhibition of structural development of the chloroplast. For laevulinic acid to effectively inhibit haem synthesis destined for NR protein synthesis when the protohaem pool in the greened barley leaves is likely to be relatively high, then protohaem must be turned over fairly rapidly as proposed by Castelfranco and Jones (1975).

Effect of Laevulinic Acid on the NR associated NADH-CR Species Observed on Sucrose Density Gradient Analysis of Cell Free Extracts of Greening, Etiolated Barley

Cell-free extracts derived from nitrate grown barley plants have been shown to contain small NADH-CR species of 3.8 and 3.1S in addition to the 7.7S NADH-CR species which represents the intact NR complex (Small and Wray, 1980a). Since these small NADH-CR species were shown by Small and Wray to be completely absent from cell free extracts derived from nitrate-less plants it appears that they were 'induced' by nitrate together with the NR complex. These small NADH-CR species were suggested by Small (1980) to be derived from NR as the partially purified NR in a sample of Biogel A1.5 m eluent (which would be expected to be free from small NADH-CR species) appeared to break down on sucrose density gradients to 3.8 and 3.1S NADH-CR species. It has been suggested that these small NADH-CR species might be dissociation or degradation products of the NR complex or precursor moieties of NR. It was hoped that by estimating the levels of 7.7S and smaller NADH-CR species in cell-free extracts of laevulinic acid treated plants and investigating the partial activities of NR associated with the 7.7S peak information could be obtained regarding the affect of haem deficiency on synthesis and/or assembly of the functional NR complex.

Cell-free extracts derived from cut shoots which had been maintained for 24 h in the absence and presence of

30mM laevulinic acid (as previously described for the time course experiment) were subjected to sucrose density gradient analysis (Fig.9a and b respectively).

The proportions of NADH-, $FMNH_2$-, MVH-NR and NADH-CR activities in the 7.7S peak of the laevulinic acid treatment compared with the control were 65%, 54%, 57% and 71% respectively. Since a similar amount of all of the partial activities of NR were lost in the presence of laevulinic acid it follows that there would appear to be total loss of NR complex due to a requirement for haem in assembly or synthesis of NR. This assumes that NADH-CR, $FMNH_2$-NR and MVH-NR activities do not require haem for electron transport or for the correct conformation to get activity. Although the non-involvement of haem in MVH-NR activity is thought likely (see Introduction), there is dispute as to whether cyt b_{557} is involved in NADH-CR activity and doubt as to whether haem is required for $FMNH_2$-NR activity. If haem was required for these activities then one would expect the proportions of the NADH-CR and $FMNH_2$-NR activities to be lower than MVH-NR activity, compared with the control, but this was not the case.

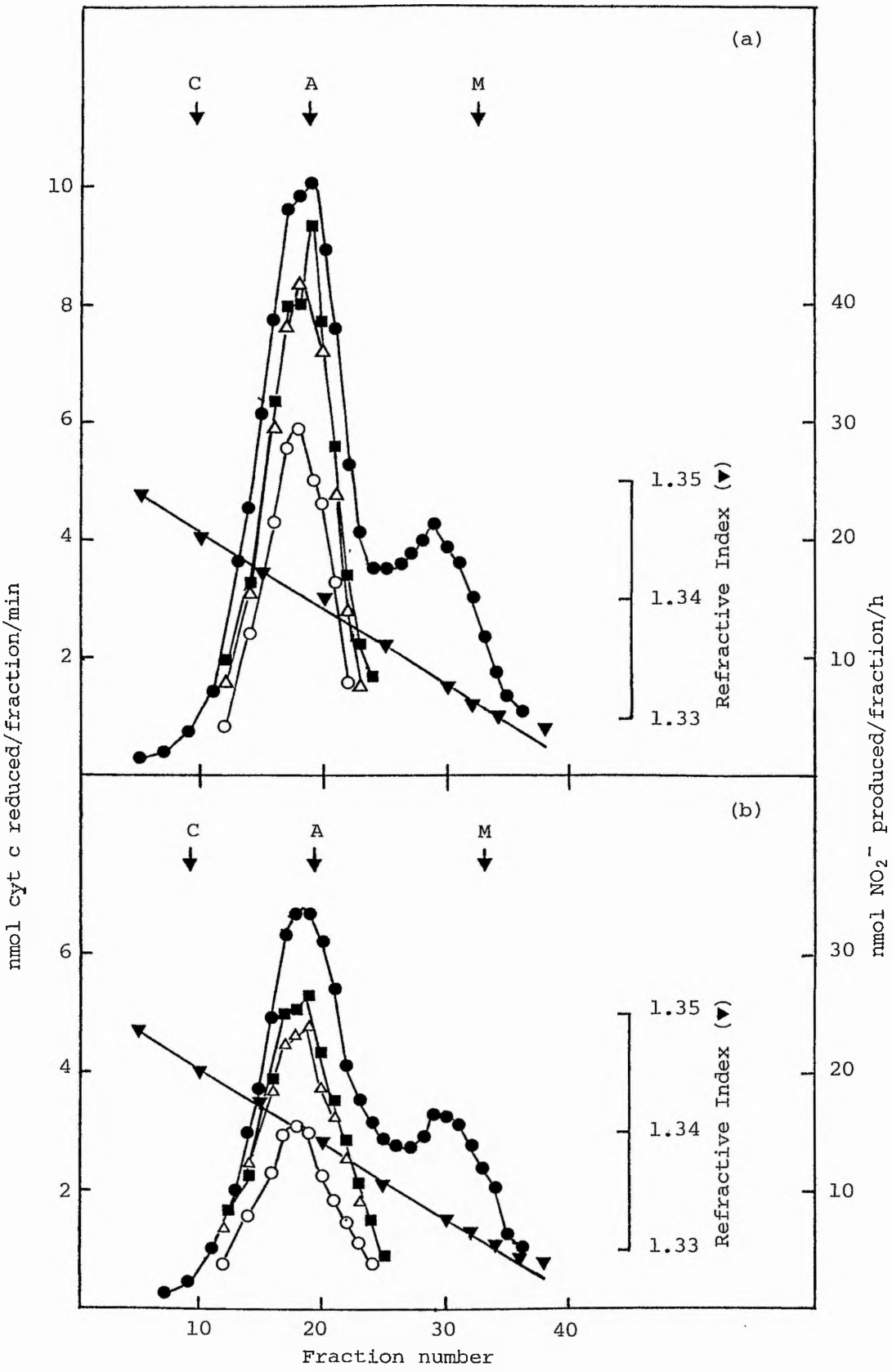
The levels of both the 7.7S peak and the small NADH-CR peak were reduced in the laevulinic acid treatment and the ratios of small to large NADH-CR species were approximately the same for laevulinic acid treatment and the control. It has been suggested that the small NADH-CR species are

Fig.9

Sucrose Density Gradient Centrifugation Analysis of Extracts from Greening, Etiolated Barley Shoots Which Had Been Maintained in the Absence and Presence of Laevulinic Acid

Barley seedlings were grown in the dark at 28 °C for 3 days and watered daily with half-Hoagland nutrient solution lacking nitrate. The shoots were cut under water, the coleoptiles removed, and placed into beakers (20/beaker) containing 100mM KNO_3 and 50mM KH_2PO_4, pH 7.0 with no additions (a) or 30mM laevulinic acid (b). After 4 h pre-incubation in the dark the shoots were illuminated for 24 h after which time they were removed from the beakers, rinsed with distilled water, blotted dry and weighed. Shoots were extracted in Buffer I (3 ml buffer/ g FW tissue), filtered through muslin and centrifuged at 38 000 g for 20 minutes.

This figure shows the distribution of NADH- (■), $FMNH_2$- (△), MVH-NR (o) and NADH-CR (●) activities following analysis of 0.4 ml aliquots of each sample on 2-18% sucrose gradients. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
C
A
M
10
8
6
4
2
40
30
20
10
1.35
1.34
1.33
Refractive Index (▼)
(b)
C
A
M
6
4
2
30
20
10
1.35
1.34
1.33
Refractive Index (▼)
10
20
30
40
Fraction number
nmol cyt c reduced/fraction/min
nmol NO_2^- produced/fraction/h

precursors of NR. If these proposed precursors could be synthesised in the absence of haem then lack of haem does not affect assembly of these precursors into the NR complex since there is clearly no build up of small NADH-CR species in the presence of laevulinic acid (Fig.9b). An alternative explanation for the origin of the small NADH-CR species is that they represent relatively stable breakdown products of NR. If essentially the same NR complex was formed in the absence of haem as in the presence, but was turned over rapidly because it was incomplete, then one would again expect to observe a build-up of these smaller NADH-CR species, but this was not the case.

It was assumed that as NR activity was observed in extracts of laevulinic acid treated barley shoots, the level of laevulinic acid used in these studies (30mM) was not enough to prevent sufficient haem synthesis to allow substantial production of the NR complex, thus complicating the interpretation of results.

Although the effect of laevulinic acid on the assays of NR activities *per se* was not investigated here or reported by Nasrulhaq-Boyce and Jones (1977) it has, however, been shown that nitrate accumulated in the laevulinic acid treated shoots (Fig.7f). This would tend to suggest that the inhibition of NR activities was due to impaired production of a functional NR complex and was not merely an artefact produced by laevulinic acid.

The findings of Nasrulhaq-Boyce and Jones (1977) that NADH-NR, MVH-NR and NADH-CR activities were similarly inhibited by laevulinic acid were confirmed by the work described here. In addition $FMNH_2$-NR activity was also shown to be similarly inhibited by laevulinic acid. This is of importance since it has been suggested that $FMNH_2$ and MVH may donate electrons to different portions of the NR molecule and that $FMNH_2$ may be capable of donating electrons directly to the haem moiety (see Introduction). The inhibition of all activities of NR by laevulinic acid provoked Nasrulhaq-Boyce and Jones (1977) to suggest that synthesis of NR is an integrated process and if haem is lacking the protein is not synthesised.

Support for this hypothesis is provided by the analysis of laevulinic acid treated tissue extracts by sucrose density gradient analysis described in this thesis. In contrast to the situation in molybdenum-deficit when essentially the same complex is formed in the absence as in the presence of molybdenum (Wray and Filner, 1970) it would appear that in haem deficiency NR holoenzyme production is reduced since all NR activities associated with the 7.7S peak were reduced. If the small NADH-CR species observed on sucrose gradients represent precursor NR proteins or apo-NR then haem deficiency also affects their synthesis since no build-up of these species was observed in extracts of laevulinic acid treated tissue. On the other hand, if the NADH-CR species represent NR breakdown products, the lack of build-up of these species could be interpreted

to mean that haem-deficiency does not produce a more labile holoenzyme.

An early finding in this study was that NR is unstable in crude extracts, especially extracts derived from older leaf tissue. It is therefore clear that the analysis of the sucrose gradient data described here must be of a speculative nature. This is especially true of conclusions regarding the small NADH-CR species which are also likely to be unstable *in vitro* (see Chapter 2). In addition interpretation is largely dependent on the origin of the small NADH-CR species and it is to this end that much of the remainder of this thesis is devoted.

CHAPTER 2

AGE-DEPENDENT *IN VITRO* STABILITY OF NITRATE REDUCTASE FROM BARLEY LEAVES

Amount and Stability of NR Extracted from Primary Leaves of 4, 5 and 6 Day Old Barley Leaves

The amount of NR activity which can be detected by a 10 min assay following tissue extraction and centrifugation of extract for 20 min is shown in Table 3 (column a). Maximum amounts of NR activity were found in extracts from 4 day old primary leaves and the NR activity decreased with increasing leaf age.

However, the results presented in Table 3 (column a) may not reflect the amount of NR present in the tissue at the time of extraction since 40 min elapsed between the start of the extraction procedure and the end of the assay. The progressive loss of activity with age could be due to the development of some type of age-dependent inactivating mechanism which operates subsequent to extraction. The processing period was therefore reduced to 15 min by omitting the centrifugation step. Tissue was extracted, filtered through muslin and assayed for 10 min. The NR activities obtained are shown in Table 3 (column b). Although the NR activity present in extracts from 4 day old leaves did not differ from that obtained if a centrifugation step was employed, the activity found in extracts from 5 and 6 day old leaves was much higher. This higher activity may be a consequence of the reduced time available for the postulated inactivating mechanism to operate.

Table 3

NR Activity Detected in Centrifuged and Uncentrifuged Extracts of Barley Primary Leaves of Different Ages

Age of barley primary leaf (day)	NR activity (µmol nitrite produced/ml extract/h	
	(a) centrifuged	(b) uncentrifuged
4	2.69	2.93
5	1.92	2.70
6	0.90	1.76

Primary leaves were extracted with Buffer I (1 g tissue/5 ml buffer) and the brei was either centrifuged at 38 000 g for 20 min or filtered through muslin. 0.1 ml aliquots were then assayed for NR activity for 10 min.

The stability of NR in uncentrifuged leaf extracts was determined by maintaining the extract at 4°C and removing aliquots at time intervals for assay (Fig.10). NR present in extracts from 4 day old primary leaves was most stable but still lost 10% of the initial activity over a 2 h period. NR in extracts from 4 day old primary leaves had a half life of 358 min while the half life of NR in extracts from 5 and 6 day old primary leaves was 107 and 70 min respectively. The decreased stability of NR in extracts from primary leaves older than 4 days suggests that the postulated inactivating mechanism becomes increasingly effective as the leaf ages or that NR becomes more susceptible to inactivation.

Age Dependent Conversion of NR to Smaller NADH-CR Species

Sucrose density gradient centrifugation analysis of extracts from 4 day old primary leaves is shown in Fig.11a. The major NADH-CR species co-sediments with NR activity at 7.7S whilst the small amount of NADH-CR activity in the 3-4S region of the gradient represents NADH-CR species which have been shown to be possibly derived from NR (Small, 1980) in addition to a constitutive NADH-CR species.

Sucrose density gradient centrifugation analysis of extracts from 5 day old leaves showed a different profile of NADH-CR activity (Fig.11b). The NADH-CR species sedimenting in the 3-4S region were more predominant and heterogeneous, whilst the amount of the 7.7S NR complex

Fig.10

Stability at 4 °C of NR Present in Extracts from 4 Day (●), 5 Day (▲) and 6 Day (■) Old Primary Leaves

Primary leaves were extracted in Buffer I and the brei was filtered through a double layer of muslin. The extracts were maintained at 4 °C and aliquots were removed at the times indicated for assay of NR activity.

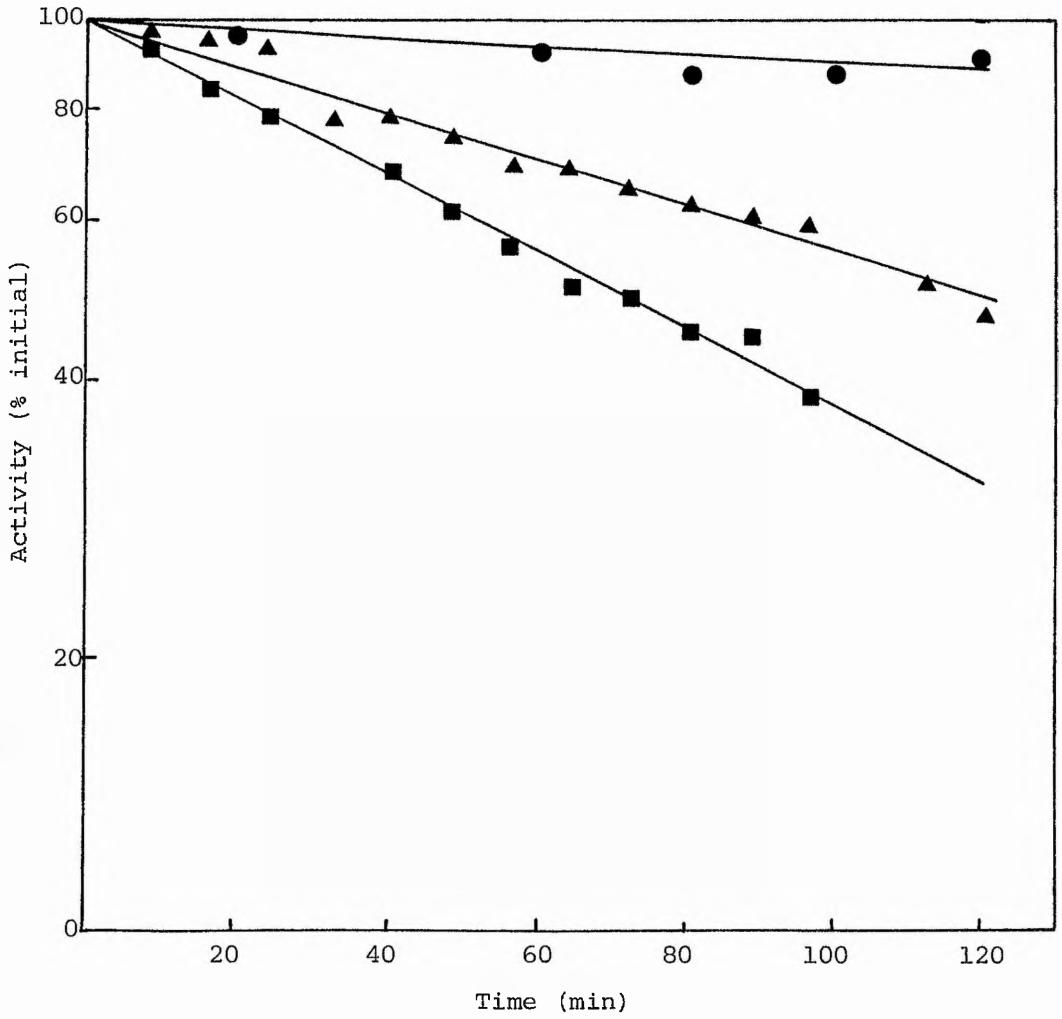
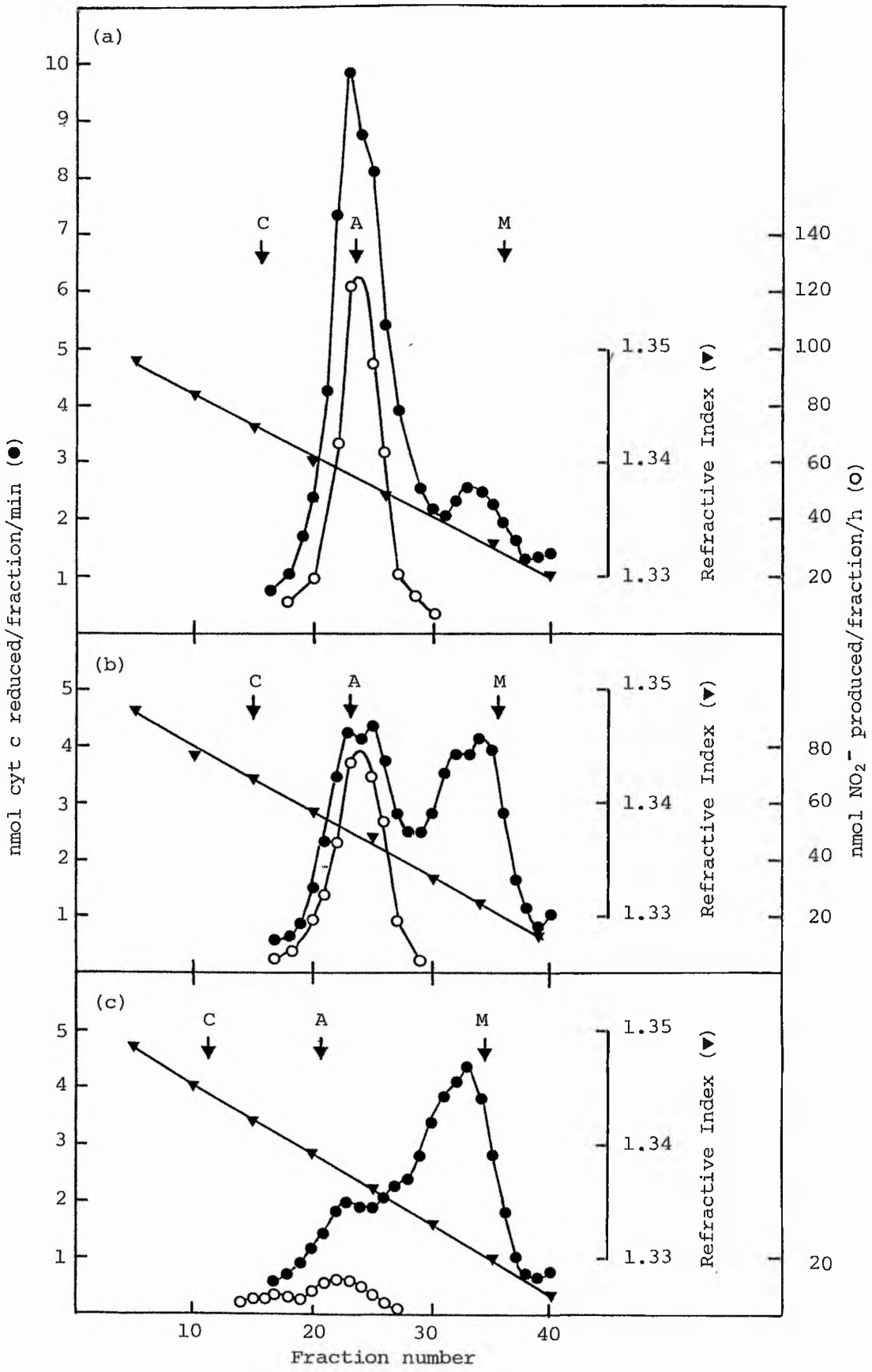

100
80
60
40
20
0
Activity (% initial)
20
40
60
80
100
120
Time (min)

Fig.11

Sucrose Density Gradient Centrifugation Analysis of Extracts from 4, 5 and 6 Day Old Primary Leaves

Primary leaves were extracted in Buffer I and the brei was centrifuged at 38 000 g for 20 min. The NADH-NR activities of the 4, 5 and 6 day old primary leaf extracts were 1.68, 1.46 and 1.06µmol nitrite produced/ml extract/h respectively.

This figure shows the distribution of NADH-NR(o) and NADH-CR (•) activities after analysis of 0.4 ml aliquots of the 4 (a), 5 (b) and 6 (c) day old plant extracts on 2-18% sucrose gradients. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
(b)
(c)
C
A
M
nmol cyt c reduced/fraction/min (●)
Refractive Index (▼)
nmol NO_2^- produced/fraction/h (○)
1.35
1.34
1.33
Fraction number
10
20
30
40

present was decreased. There was also an additional NADH-CR species present which sedimented at 6.8S and also possessed NR activity.

Sucrose density gradient analysis of extracts from 6 day old primary leaves (Fig.11c) showed that the major NADH-CR species present sedimented in the 3-4S region while very small amounts of the 7.7S and 6.8S NR-associated NADH-CR species were also present.

Analysis resolved two major species in the 3-4S region of the gradient sedimenting at 3.1S and 3.8S and it seems likely that these species, in addition to the 6.8S NADH-CR species, represent breakdown products of NR. As the amount of these breakdown products is greater in the extracts from older tissue one might conclude that the age-dependent instability is associated with an increased conversion of the NR complex to these smaller NADH-CR species.

Correlation of *in vitro* Stability of NR with Leaf Position

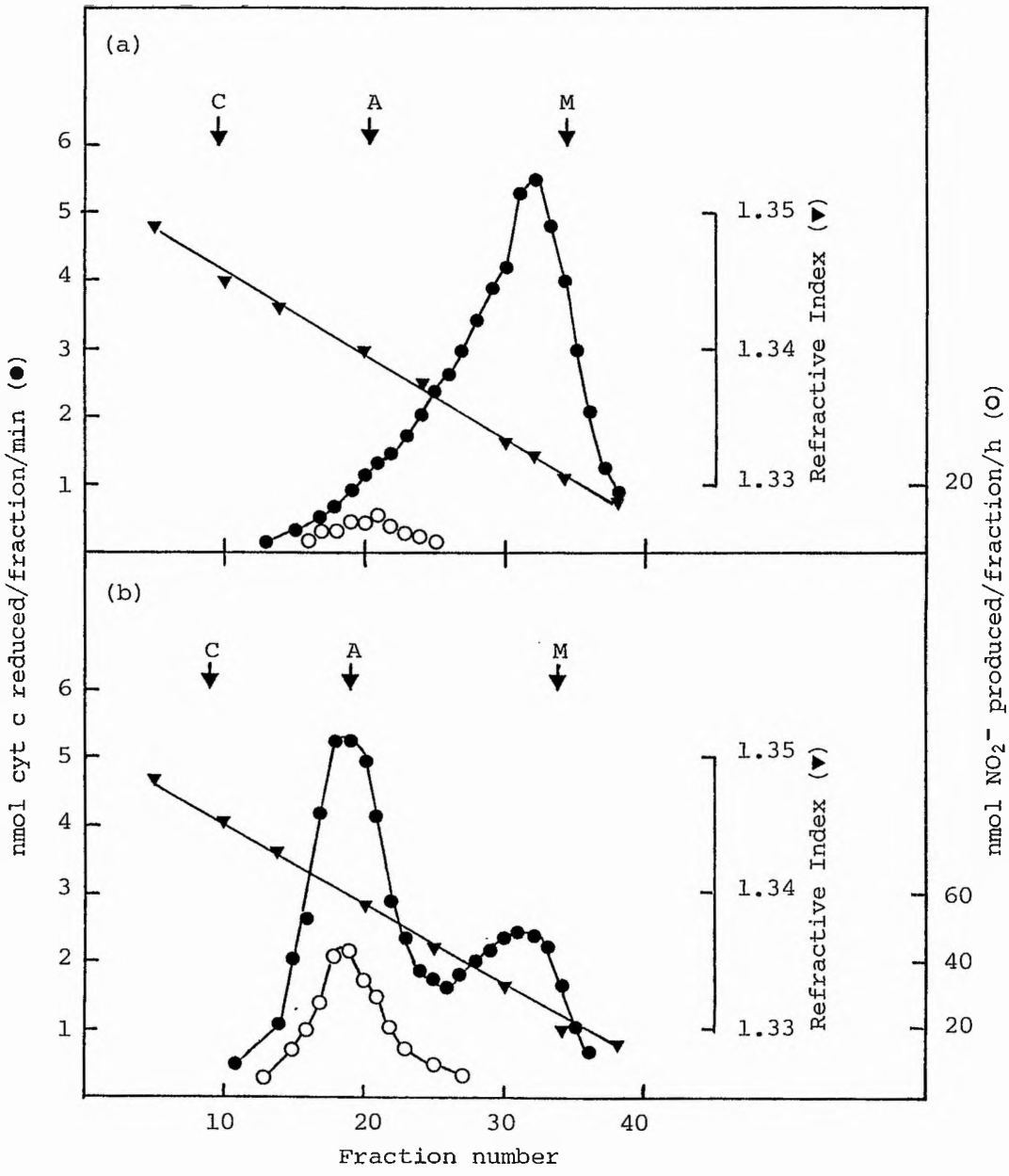
Sucrose density gradient analysis of extracts of primary and secondary leaves from 6 day old plants is shown in Fig.12a and Fig.12b respectively. As was previously shown, the major NADH-CR species in the primary leaf extract sedimented in the 3-4S region of the gradient representing the presence of highly unstable NR in this older leaf tissue. However, the major NADH-CR species in the secondary leaf extract co-sediments with the NR activity at 7.7S thus representing a more stable NR complex

Fig.12

Sucrose Density Gradient Centrifugation Analysis of Extracts from 6 Day Old Primary Leaves and 6 Day Old Secondary Leaves

Leaves were extracted in Buffer I and the brei was centrifuged at 38 000 g for 20 min. The NADH-NR activities of the primary and secondary leaf extracts were 0.45 and 1.12µmol nitrite produced/ml extract/h respectively.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after analysis of 0.4 ml aliquots of the primary leaf (a) and secondary leaf (b) extracts on 2-18% sucrose gradients. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
C
A
M
6
5
4
3
2
1
1.35
1.34
1.33
Refractive Index (▼)
20
(b)
C
A
M
6
5
4
3
2
1
1.35
1.34
1.33
Refractive Index (▼)
60
40
20
10
20
30
40
Fraction number
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)

in this younger tissue, the pattern of NADH-CR activity more resembling that from 4 day old primary leaf extract.

Thus, different degrees of age-dependent inactivation would appear to occur within the same barley plant.

Amount and Stability of Nitrite Reductase in Primary Leaf Extracts of 4, 5 and 6 Day Old Barley Plants

The possibility that the phenomenon of age-dependent instability is extended to the next enzyme in the nitrate assimilation pathway was investigated.

Centrifuged extracts of 4, 5 and 6 day old primary leaves were maintained at 4 °C and aliquots removed at time intervals for assay of nitrite reductase activity as described in Methods (Table 4).

In contrast to NR, nitrite reductase activity was highest in the 6 day old primary leaf extract. In addition there would appear to be no major differences in the stability of nitrite reductase with respect to tissue age although accurate evaluation of stability was complicated by the increase in nitrite reductase activity in the extracts after 1 h at 4°C.

However, subsequent work by I.S. Small (personal communication) has confirmed that nitrite reductase does not exhibit age-dependent *in vitro* stability, at least in wheat.

Table 4

The Amount and Stability of Nitrite Reductase Present in Centrifuged Extracts of Barley Primary Leaves of Different Ages

Age of barley primary leaf (d)	Nitrite reductase activity initially present in extracts (µmol nitrite reduced/ml extract/h	Nitrite reductase activity (% of initial remaining after		
		0 h	1 h	2 h
4	4.17	100	105	83
5	5.00	100	148	91
6	6.30	100	122	63

Primary leaves were extracted with Buffer I and the brei was centrifuged at 38 000 g for 20 min. The extracts were maintained at 4°C and nitrite reductase activity measured at the times indicated.

Effect of BSA on the Amount and Stability of NR Extracted from 4, 5 and 6 Day Old Primary Leaves of Barley

Age dependent instability of NR has been observed in leaves of oats, tobacco and maize (Schrader *et al*, 1974a; 1974b) cotyledons of cotton (Purvis *et al*, 1976; Tischler *et al*, 1978) and roots of maize (Oaks *et al*, 1972; Wallace, 1973b, 1975b). In all of these cases the presence of non-plant protein, either BSA or casein, in the extraction medium increased the level of extractable NR from the older tissue and the stability of the extracted enzyme. The effect of BSA on the barley leaf system was therefore investigated.

Inclusion of 3% BSA in the extraction buffer did not increase the amount of NR activity obtained after a 10 min assay following centrifugation of extracts from 4 and 5 day old primary leaves. However it markedly increased the NR activity in extracts from 6 day old primary leaves (Table 5). The stability of NR in extracts from 4 day old primary leaves maintained at 4°C was not affected by the presence of BSA in the extraction buffer (Table 5) suggesting that BSA is not able to protect NR from the small amount of inactivation which occurs at this time. In contrast, the stability of NR in extracts from 5 and 6 day old primary leaves was increased when BSA was present in the extraction buffer. In addition, NR in extracts of 5 and 6 day old primary leaves prepared in the presence of BSA had the same stability as NR in extracts of 4 day old primary leaves prepared without BSA.

Table 5

Effect of BSA on the Amount and Stability of NR Present in Centrifuged Extracts of Barley Primary Leaves of Different Ages

Age of barley primary leaf (d)	BSA	NR activity initially present in extract (µmol nitrite produced/ml extract/h)	NR activity (% of initial remaining) after		
			0 h	2 h	4 h
4	-	2.34	100	89.3	71.8
	+	2.42	100	84.9	71.2
5	-	2.33	100	50.6	30.0
	+	2.38	100	87.2	76.7
6	-	0 90	100	32.6	17.8
	+	1.96	100	85.3	75.0

Primary leaves were extracted in Buffer I containing either no additions or 3% BSA and the brei was centrifuged at 38 000 g for 20 min. The extracts were maintained at 4 °C and NR activity measured at the times indicated.

These results suggest that extracts from 4, 5 and 6 day old primary leaves possess an inactivating mechanism which cannot be blocked by BSA and that extracts from 5 and 6 day old primary leaves possess an additional inactivating mechanism which can be blocked by BSA.

Effect of BSA on the Amount of NR Extracted from Primary, Secondary and Tertiary Leaves of Eighteen Day Old Field Grown Barley

To see whether the age-dependent *in vitro* stability of NR also occurred in field grown plants, barley was grown during summer 1979 as detailed in the Methods Section I.

Eighteen days after sowing, the first, second and third leaf blades were removed and extracted in Buffer I with or without BSA. After centrifugation, most NR activity was detected in the extract from the third leaf blade (i.e. the youngest) and negligible NR activity was detected in the extracts from the older leaf tissue if BSA was not present during extraction (Table 6). Since BSA was able to stabilise NR activity in the older leaves it would appear that age-dependent *in vitro* instability is not merely a laboratory phenomenon.

Effect of BSA on the Age-Dependent Conversion of NR to Smaller NADH-CR Species

Earlier in this chapter it was observed that age-dependent stability of NR correlated with an increased conversion of NR to smaller NADH-CR species. To determine

Table 6

NR Activity Present in Extracts from Leaves of 18 Day Old Field Grown Barley Plants

Leaf	BSA	NR activity (µmol nitrite produced/ ml extract/h)
First (Oldest)	-	0.01
	+	0.27
Second	-	0.07
	+	0.36
Third (Youngest)	-	0.34
	+	0.42

Leaves were extracted with Buffer I or Buffer I containing 3% (w/v) BSA. The brei was centrifuged at 38 000 g for 20 min and NR activity of the extract estimated.

whether BSA decreases the conversion of NR to smaller CR species as well as stabilising NR, 6 day old primary leaves were extracted either in the absence of BSA or the presence of BSA and subjected to sucrose density gradient analysis (Fig.13a and b respectively).

The major NADH-CR species in the BSA treated extract sedimented at 7.7S with NR activity and the conversion to smaller NADH-CR species appeared to be reduced.

It is not known whether the small amount of NADH-CR species sedimenting in the 3-4S region of the BSA treatment are actually present *in vivo*, whether they are produced before BSA acts or whether they represent products of an inactivation mechanism which cannot be blocked by BSA.

The Effect of Delayed Addition of BSA on the Amount and Stability of NR in Extracts from Barley Primary Leaves of Different Ages

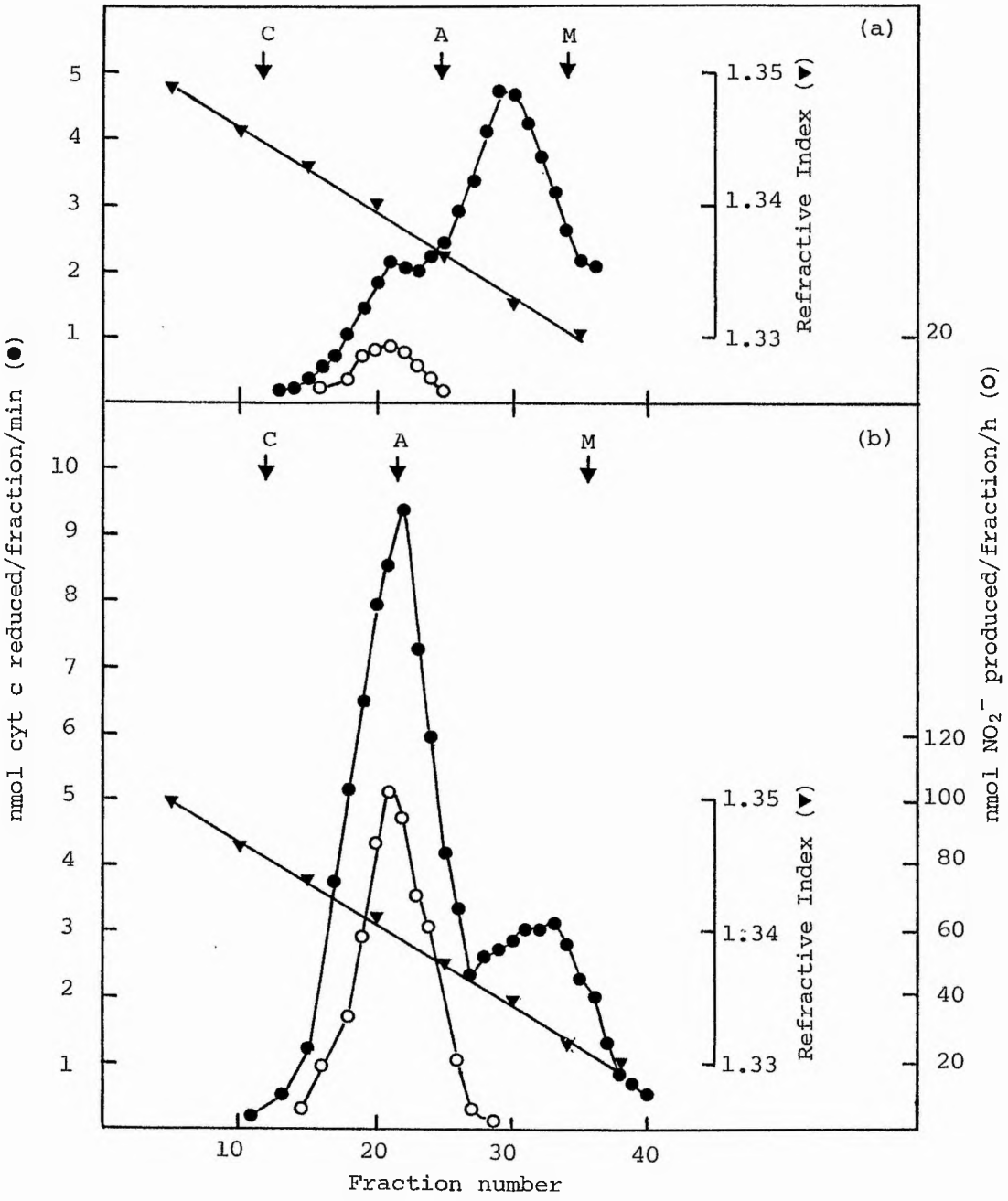
Purvis *et al* (1976) observed that delayed addition of BSA to cotton cotyledon extracts was as effective in increasing NR activity as when it was added immediately after extraction. This suggested to them that NR is inactivated at the same rate in the presence or absence of BSA and therefore concluded that the major effect of BSA on NR activity in cotton cotyledon extracts was to make the extracted enzyme more active rather than increase the amount of NR extracted or improve the stability of

Fig.13

Sucrose Density Gradient Centrifugation Analysis of Extracts from 6 Day Old Primary Leaves Prepared in the Presence and Absence of BSA

6 day old primary leaves were extracted with Buffer I containing either no additions or 3% (w/v) BSA. After centrifugation of the brei at 38 000 g for 20 min NR activities of the extracts were 0.91 and 2.27µmol nitrite produced/ml extract/h respectively.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after analysis of 0.4 ml aliquots of the extracts prepared in the absence (a) and the presence (b) of BSA on 2-18% sucrose gradients. C, A and M denote the relative positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
(b)
C
A
M
1.35
1.34
1.33
Refractive Index (▼)
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)
Fraction number
10
20
30
40

extracted enzyme.

The effect of delayed addition of BSA on NR activity of primary leaf extracts of barley plants of different ages was therefore examined (Table 7).

Delayed addition of BSA significantly stabilised NR activity, especially in the extracts from older tissue. However, in contrast to the conclusions of Purvis *et al* (1976) delayed addition of BSA was found not to be as efficient in stabilising NR as BSA present from the time of extraction.

On analysis of the results obtained by Purvis *et al* (1976 Table 1) it is clear that they have misinterpreted their own data. Despite noting that NR in extracts of cotton cotyledons became less stable on dilution, NR activity in extracts diluted (1:1) at time zero with Buffer +4% BSA was compared directly with activity in extracts which had been maintained in the more concentrated form until delayed addition of buffer +4% BSA.

It would therefore seem feasible that delayed addition of BSA prevents any further inactivation of NR by the proposed inactivating system in cell-free extracts but

Table 7

Effect of Delayed Addition of BSA on the Amount and Stability of NR in Extracts of Barley Primary Leaves of Different Ages

Age of barley primary leaf (d)	BSA	NR activity present in the extract before centrifugation (µmol nitrite produced/ ml extract/h)	NR activity (% of initial remaining) after	
			0 min	40 min
4	-	2.94	100	94.4
	+	3.00	100	97.5
	Delayed	3.03	100	99.4
5	-	1.61	100	59.1
	+	3.04	100	98.6
	Delayed	2.14	100	70.0
6	-	0.45	100	54.6
	+	2.63	100	97.9
	Delayed	0.66	100	62.2

Primary leaves were extracted with Buffer I containing either no additions or 3% BSA. Solid BSA was added to an aliquot of the filtered control extract to give a final concentration of 3% (w/v). After centrifugation at 38 000 g for 20 min the extracts were maintained at 4°C and NR activity measured at the times indicated after delayed addition of BSA.

is unable to recover NR activity lost before BSA is added.

Effect of Delayed Addition of BSA on the Age-Dependent Conversion of NR to Smaller NADH-CR Species

If delayed addition of BSA protects NR from further inactivation one might expect this to be accompanied by a decrease in conversion of NR to smaller NADH-CR species. This was found to be the case (Fig.14c) with the NADH-CR breakdown profile of the delayed addition treatment being intermediate between the minus and plus BSA treatments.

Such data does not rule out the possibility that BSA increases NR activity as postulated by Purvis *et al* (1976) since estimation of specific activity of NR on the gradients was made impossible by the use of BSA. However, one can see that there is a greater amount of small NADH-CR species with the delayed addition treatment (Fig.14c) than with the plus BSA treatment (Fig.14b) suggesting that breakdown of NR has occurred in the former to a greater extent.

The Effect of Endogenous Protein Concentration on Stability of NR

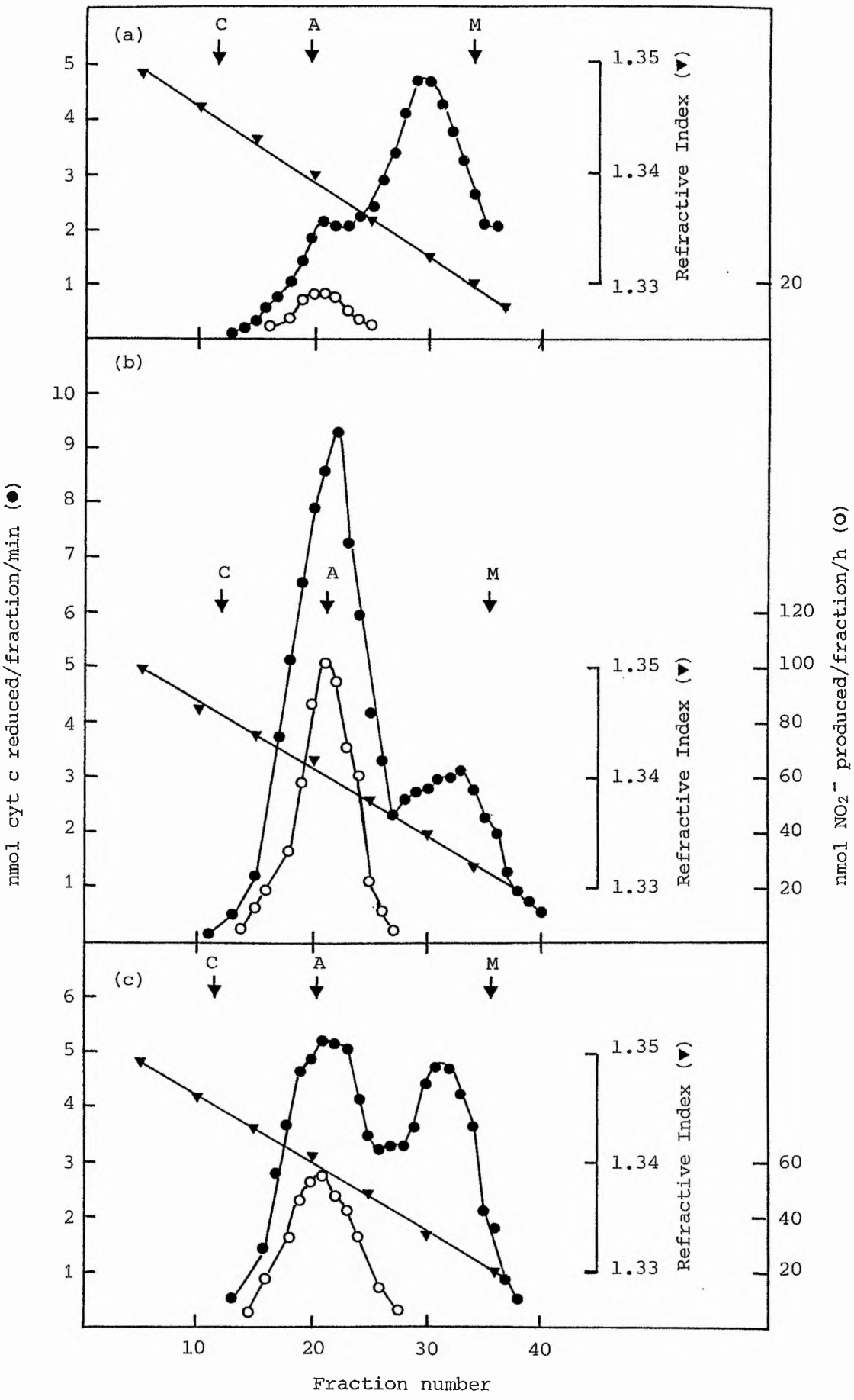
It has been suggested that protein concentration may be important in maintaining stability of NR since dilution of extracts from cotton cotyledons resulted in a more labile enzyme (Tischler *et al*, 1978) while the high protein

Fig.14

Sucrose Density Gradient Centrifugation Analysis of Extracts from 6 Day Old Primary Leaves Prepared in the Absence and Presence of BSA and also Extract to which Addition of BSA was Delayed

6 day old primary leaves were extracted with Buffer I containing either no additions or 3% BSA. Solid BSA was also added to an aliquot of filtered extract to give a final concentration of 3%. After centrifugation at 38 000 g for 20 min NR activities of the extracts were 0.91, 2.27 and 1.44µmol nitrite produced/ml extract/h.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after 0.4 ml aliquots of the extracts prepared in the absence (a) and presence (b) of BSA and extract to which addition of BSA was delayed (c) were analysed on 2-18% sucrose gradients. C, A and M denote the respective positions of catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
(b)
(c)
C
A
M
1.35
1.34
1.33
Refractive Index (▼)
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)
Fraction number
10
20
30
40

concentrations in the presence of exogenous protein might prevent dissociation of the NR complex (Schrader *et al*, 1974a; Sherrard and Dalling, 1978).

However, NR activity in extracts from 4, 5 and 6 day old primary leaf extracts did not correlate with the protein content of the extract (Table 8) and the stability of NR in 4 day old primary leaf extract was not decreased by dilution (Table 9) but rather increased.

To illustrate this further an extract of 4 day old primary leaves was diluted to be comparable with 6 day old primary leaf extract with respect to NR activity and was compared to undiluted 4 day old primary leaf extract by sucrose density gradient analysis (Fig.15). The pattern of NADH-CR activity resulting from the diluted 4 day old leaf extract remained essentially the same as that from the undiluted extract.

These results suggest that the concentration of endogenous protein *per se* does not contribute to NR stability in crude extracts.

Does BSA Stabilise by binding to NR?

Dalling *et al* (1972) reported that NR binds to ruptured cell organelles only when BSA is absent from the extraction buffer. It has therefore been suggested that BSA might protect NR from a binding inactivator by binding directly to the NR complex. Such binding would presumably occur at some site other than the active site since

Table 8

NR Activity and Protein Present in Centrifuged Extracts of Barley Primary Leaves of Different Ages

Age of barley primary leaf (d)	Protein (mg/ml extract)	NR activity (µmol nitrite produced/ml extract/h)
4	6.74	2.61
5	8.43	2.14
6	6.87	0.59

Primary leaves were extracted in Buffer I and the brei was centrifuged at 38 000 g for 20 min. NR activity and protein was estimated by the methods described in the Methods Section.

Table 9

Effect of Dilution on Stability at 4°C of NR in Extracts of 4 Day Old Primary Leaves of Barley

Time at 4°C (min)	NR activity (% of initial remaining after the times indicated) in extracts -			
	Undiluted (1:0)	Diluted (3:1)	Diluted (1:1)	Diluted (1:3)
0	100	100	100	100
20	95.2	96.4	94.4	100.3
40	95.6	100.8	100	107.2
60	94.2	101.9	98.4	107.5
80	87.9	98.7	100.4	101.3
100	89.8	98.6	96.4	104.6
120	86.5	95.3	90.5	106.2

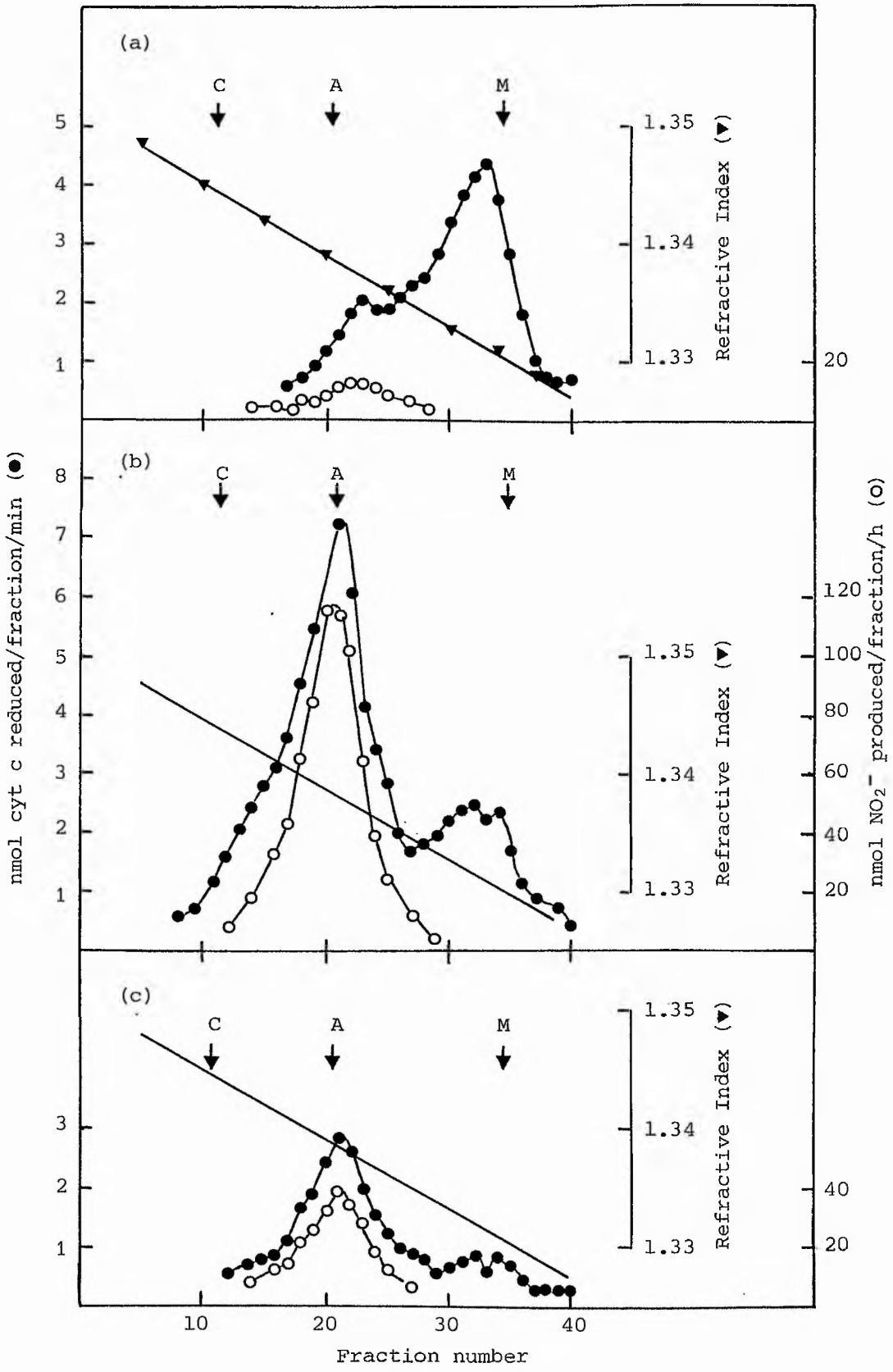
Primary leaves were extracted in Buffer I and the brei was centrifuged at 38 000 g for 20 min. Buffer I was used to dilute the extract by the appropriate factor and loss of NR activity at 4°C was measured.

Fig.15

Sucrose Density Gradient Centrifugation Analysis of Extracts of 6 Day Old Primary and Secondary Leaves and 4 Day Old Primary Leaves Diluted with Buffer I

4 and 6 day old primary leaves were extracted with Buffer I. After centrifugation of the brei at 38 000 g for 20 min NR activities of the extracts were 1.98 and 0.37µmol nitrite produced/ml extract/h respectively.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after analysis of 0.4 ml aliquots of the 6 day old primary leaf extract (a), the 4 day old primary leaf extract (b) and 4 day old primary leaf extract diluted to be comparable with 6 day old extract with respect to NR activity (c) on 2-18% sucrose gradients. C, A and M denote the relative positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
(b)
(c)
C
A
M
nmol cyt c reduced/fraction/min (●)
Refractive Index (▶)
nmol NO_2^- produced/fraction/h (○)
1.35
1.34
1.33
Fraction number
10
20
30
40

NADH-NR activity is stabilised in the presence of BSA. However one might expect $FMNH_2$-NR and/or MVH-NR activities to be affected in the presence of BSA if, as has been suggested, $FMNH_2$ and MVH donate electrons to different sites on the NR complex. The effect of BSA on the partial activities of the NR complex was therefore assessed in 6 day old primary leaf extracts (Table 10).

The $FMNH_2$-NR and MVH-NR activities are known to be more stable than the NADH-NR activity of the complex (Wray and Filner, 1970) and this was confirmed here. In addition, the presence of BSA was not found to improve the stability of the already more stable NR activities.

Since the initial $FMNH_2$-NR and MVH-NR activities of the extract were similar in the absence and presence of BSA it would appear that if BSA binds to NR it does not affect the accessibility of the sites of electron donation to any great extent.

Effect of PMSF on the Amount and Stability of NR from Primary Leaves of Different Ages

It is likely that on extraction, proteinases are released from the vacuole and possibly other compartments in the cell resulting in degradation of NR. It has therefore been suggested that BSA might stabilise NR by acting as an alternative substrate for proteinases.

At the time this work was carried out there was no evidence for the existence of a NR-specific proteinase in

Table 10

Effect of BSA on the Partial Activities of NR in Centrifuged Extracts of 6 Day Old Primary Leaves of Barley

Electron Donor	BSA	NR activity initially present in extract (µmol nitrite produced/ ml extract/h)	NR activity (% of initial remaining) after			
			0 min	30 min	60 min	90 min
NADH	-	0.68	100	82.6	68.3	55.9
	+	0.85	100	91.3	85.0	70.5
FMNH	-	1.00	100	75.4	80.8	71.1
	+	1.39	100	82.6	92.5	72.2
MVH	-	0.36	100	130.8	92.9	74.6
	+	0.40	100	114.5	83.3	76.1

Primary leaves of 6 day old barley plants were extracted in Buffer I containing either no additions or 3% BSA. After centrifugation at 38 000 g for 20 min the extracts were maintained at 4 °C and aliquots removed at the times indicated for assay of NADH-, $FMNH_2$- and MVH-NR activities.

barley leaves. However, Wallace had described in some detail, a relatively NR-specific inactivator from mature maize roots and leaves. This NR inactivator was shown to have proteinase activity by its ability to degrade azocasein and was found to be PMSF-sensitive and therefore a serine proteinase. The ability of PMSF to protect barley NR from inactivation during extraction and subsequent analysis was assessed (Table 11).

1mM PMSF was much less effective than BSA in protection of NR from inactivation but did appear to retard inactivation to some extent. Higher concentrations of PMSF in the extraction buffer afforded no significant increase in NR stability (data not shown). However, it was found that the isopropanol used to dissolve PMSF was capable of stabilising NR in its own right, even when present at a concentration of 2.5% (v/v) in the extraction buffer. A typical set of data is presented in Table 12 to illustrate this effect where only 23% of the increase in NR activity in the PMSF treatment after centrifugation might be attributed to PMSF alone.

The processes of seed germination and leaf senescence have been shown to be inhibited by isopropanol and other aliphatic alcohols (Reynolds, 1977; Satler and Thimann, 1980). It has been suggested that these alcohols prevent proteolysis in these circumstances and therefore it is possible that the stabilising effect of isopropanol on NR activity may be related to this phenomenon.

Table 11

Effect of PMSF on the Amount and Stability of NR Present in Centrifuged Extracts of Barley Primary Leaves of Different Ages

Age of barley primary leaf (d)	PMSF	NR activity initially present in extract (µmol nitrite produced/ ml extract/h)	NR activity (% of initial remaining) after		
			0 h	2 h	4 h
4	-	2.34	100	89.3	71.8
	+	2.66	100	91.2	80.2
5	-	2.33	100	50.6	30.0
	+	2.10	100	61.6	38.2
6	-	0.90	100	32.6	17.8
	+	1.15	100	58.2	43.3

Primary leaves of 4, 5 and 6 day old barley plants were extracted in Buffer I containing no additions or 1mM PMSF (plus 2.5% (v/v) propan-2-ol used to dissolve PMSF). After centrifugation at 38 000 g for 20 min the extracts were maintained at 4°C and aliquots removed at the times indicated for assay of NADH-NR activity.

Table 12

Effect of Isopropanol on the Amount of NR Present in Centrifuged Extracts of 6 Day Old Primary Leaves of Barley

Treatment	NR activity initially present in extract (µmol nitrite produced/ ml extract/h)	NR activity (% of no additions treatment)
no additions	0.70	100
1mM PMSF + 5% propan-2-ol	1.04	149.4
5% propan-2-ol	0.96	138.0

Primary leaves of 6 day old barley plants were extracted in Buffer I containing no additions, 1mM PMSF + 5% isopropanol and 5% isopropanol alone. After centrifugation at 38 000 g for 20 min the extracts were assayed for NADH-NR activity.

The Effect of PMSF on the Age-Dependent Conversion of NR to Smaller NADH-CR Species

PMSF (plus isopropanol) only slightly retarded the conversion of NR to smaller CR species in 6 day old primary leaf extract subjected to sucrose density gradient analysis (Fig.16b) compared to a control (Fig.16a). PMSF is known to be highly unstable in solution and as PMSF was not included in the sucrose gradient any NR-protecting effect of PMSF in the sample applied to the gradient might be expected to diminish with time. In addition, it is likely that serine proteinases comprise a small fraction of the proteolytically active species released on extraction and that the remaining fraction may be responsible for the inactivation observed.

The Effect of Other Proteinase Inhibitors on the Amount and Stability of NR in Primary Leaf Extracts of 10 Day Old Barley Plants

As plants seem to use primary cysteine endoproteinases for protein degradation (Ryan and Walker-Simmons, 1981) 1,10-phenanthroline, an inhibitor of cysteine-dependent proteinases was assessed for its ability to stabilise NR in crude extracts of 10 day old primary leaves (Table 13). 2.5mM 1,10-phenanthroline did stabilise NR to some extent showing that cysteine endoproteinases may contribute to the instability of NR in crude extracts. Alternatively, since 1,10-phenanthroline is also a chelator of ferrous Fe, it may stabilise NR be sequestering ferrous Fe which is present in significant concentrations in phosphate buffers (B.A. Notton, personal communication).

Fig.16

Sucrose Density Gradient Centrifugation Analysis of 6 Day Old Primary Leaf Extracts Prepared in the Presence and Absence of 2.5% PMSF (+5% Isopropanol)

6 day old primary leaves were extracted with Buffer I containing either no additions or 2.5% PMSF + 5% isopropanol. After centrifugation at 38 000 g for 20 min NR activities of the extracts were 0.45 and 0.80µmol nitrite produced/ml extract/h respectively.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after 0.4 ml aliquots of the extracts prepared in the absence (a) and presence (b) of PMSF were analysed on 2-18% sucrose gradients. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.

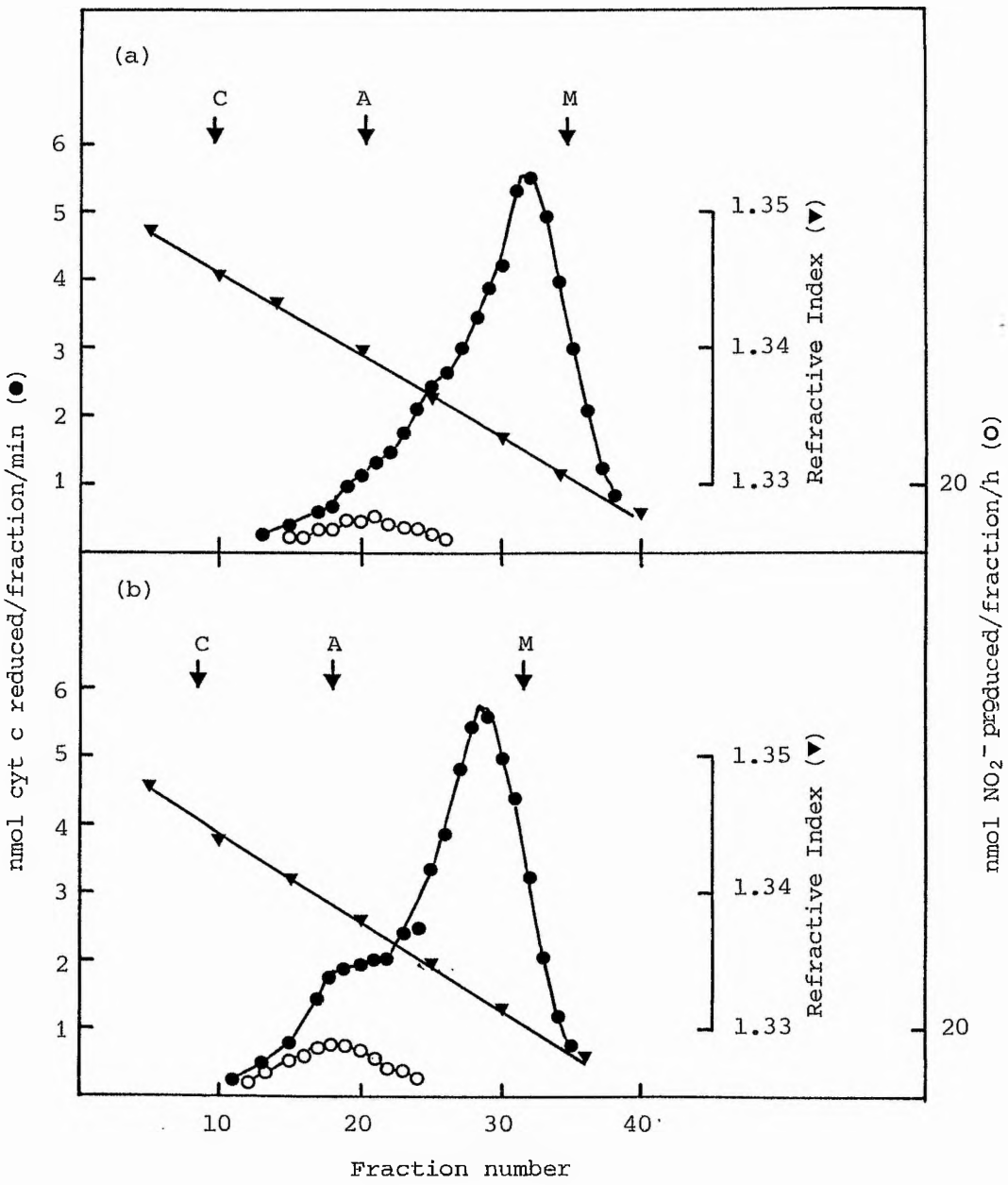

(a)
C
A
M
(b)
C
A
M
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)
Refractive Index (▼)
1.35
1.34
1.33
20
1
2
3
4
5
6
10
20
30
40
Fraction number

Table 13

Effect of 2.5mM 1,10-phenanthroline on the Amount and Stability of NR present in Centrifuged Extracts of 10 Day Old Primary Leaves of Barley

1,10-phenanthroline	NR activity initially present in extract (µmol nitrite produced/ ml extract/h)	NR activity (% of initial remaining) after		
		0 min	45 min	100 min
-	0.74	100	77	66
+	0.95	100	93	83

Primary leaves were extracted with Buffer I containing either no additions or 2.5mM 1,10-phenanthroline. After centrifugation of the brei at 38 000 g for 20 min the extracts were maintained at 4°C and aliquots removed at the times indicated for assay of NADH-NR activity.

However, combinations of PMSF, 1,10-phenanthroline and another serine proteinase inhibitor trypsin inhibitor, resulted in no major increase in the stability of NR in crude extracts (data not shown) and no combination was able to compare with the stabilising influence of BSA.

Attention must be drawn to the age of the plants used in this experiment. On using a new batch of seeds it was found necessary to prolong growth of seedlings before the age-dependent inactivation mechanism came into operation. Although a controlled investigation was not carried out it was found that seed storage conditions, especially temperature, were of great importance. The significance of these findings will be discussed with reference to work by others in the General Discussion.

Estimation of Total Proteinase Activity of Primary Leaf Extracts of Barley of Different Ages

Azocasein degrading activity of 4, 5, 6 and 7 day old primary leaf extracts were measured by a modification of the method of Charney and Tomarelli (1947) described in Methods, Section III in an attempt to correlate total proteinase activity with stability of NR.

After a 7 h incubation period with azocasein at 25°C, proteinase activity in the 4, 5, 6 and 7 day old extracts expressed as a percentage of that in the 4 day old extract were 100, 120, 124 and 113% respectively whilst after 24 h incubation activities were 100, 100, 118 and 123%. It

was concluded that the differences in activity were not significant and could not be correlated with leaf age and thus stability of NR.

With the same aim in mind, Purvis *et al* (1976) were unsuccessful in their attempts to relate the development of proteinase activity to increasing lability of NR in expanding cotton cotyledons. They therefore concluded that proteinase activity was unrelated to the *in vitro* stability of NR extracted from cotton cotyledons.

Mixing of 'Stable' and 'Unstable' Barley Leaf Extracts

Mixtures of 4 day old leaf extract, in which NR has been shown to be relatively stable, and primary leaf extract from 7 day old plants grown without nitrate, in which it was assumed the NR-inactivating system was still present, were assayed for NR activity (Table 14).

It would appear that leaf extract from 7 day old nitrate-less plants did possess factors which, when released on extraction, inactivated NR in 4 day old leaf extract. In addition, the extent of inactivation of the 'stable' NR is positively related with incubation time and amount of 7 day old extract present in the mixture (Table 14). However, the extent of inactivation was small, especially in comparison to the extent of differential lability of NR in 4 and 7 day old leaf extracts observed previously. Mixing of 4 day and 7 day nitrate-grown plant extracts also gave the approximate expected means of NR activity

Table 14

Destabilisation of NR in 4 Day Old Extracts by Mixing with 7 Day Old Extracts

Proportion of 4 day old leaf extract in the mixture (%)	NR activity expressed as $\frac{\text{actual activity}}{\text{"expected" activity}} \times 100\%$ after			
	0 h	1 h	3 h	$\bar{x}$
100	100	100	100	100
87.5	92	90	88	90
75	93	79	70	81
50	88	74	68	77
25	81	68	68	72
0	*ND	ND	ND	-
$\bar{x}$	91	82	79	

Primary leaves of 4 day old nitrate and 6 day old nitrate-less plants were extracted in Buffer I and centrifuged at 38 000 g for 20 min. 4 day extract was diluted with 7 day extract in the ratios 1:0, 7:1, 3:1, 1:1, 1:3 and 0:1. The mixtures were maintained at 4°C and aliquots removed at the times indicated for assay of NADH-NR activity. (*ND = none detactable)

(data not shown). Even if a 'specific' NR inactivation requires induction with nitrate it does not appear to be active in mixtures of crude extracts.

The inability to observe de-stabilisation of NR in mixtures of crude extracts is not surprising since NR protectors have been isolated from cotton cotyledons (Purvis *et al*, 1980) and wheat leaves (Sherrard *et al*, 1979a). Both positive and negative effectors of NR activity were isolated from wheat leaves and it was found that the activity of the negative effector could not be detected in crude extracts but only on further purification. This was presumably due to the action of the positive effector molecules in the crude extract. The significance of these findings will be analysed in the General Discussion.

Amount and Stability of NR Extracted from 4 and 8 Day Old Primary Leaves Maintained at 4 °C and 25 °C: Kuo Buffer and Buffer I Compared

A novel extraction buffer for higher plant NR has been developed by Kuo *et al* (1980). This buffer (hereafter referred to as Kuo buffer) comprises 0.25M Tris HCl pH 8.5 containing 3mM DTT, 5µm FAD, 1µM Na molybdate and 1mM EDTA. Kuo found that NR activity in 6 day old barley leaf extracts maintained at 25°C decayed at a rate of less than 3% per h using this buffer. The effect of Kuo buffer on the age-dependent instability of NR was therefore investigated in addition to its ability to stabilise NR

at the higher temperature (Fig.17).

4 day old Kuo buffer leaf extract maintained at 4°C and 25°C lost 2% and 6% respectively of the initial NR activity in 1 h compared to 6% and 73% loss in the Buffer I extracts. Similarly 8 day old primary leaf Kuo buffer extract lost only 8% of the initial NR activity in 1 h at 4°C and 17% at 25°C compared to 51% and 93% respectively for the Buffer I extracts. Thus, Kuo buffer has a remarkable effect on the stability of NR even in cell free extracts of older leaf tissue at 25°C.

One would normally expect the extract containing the more stable NR to have the higher initial activity but this was not the case (Fig.17 legend). Although this phenomenon was not investigated here, Kuo found that reduced NR activity in high concentration Tris extraction buffer could be reversed by dilution with low concentration Tris or phosphate buffers. It was therefore concluded that reduced NR activity was not due to inhibition but rather some salt effect that rendered the enzyme less active.

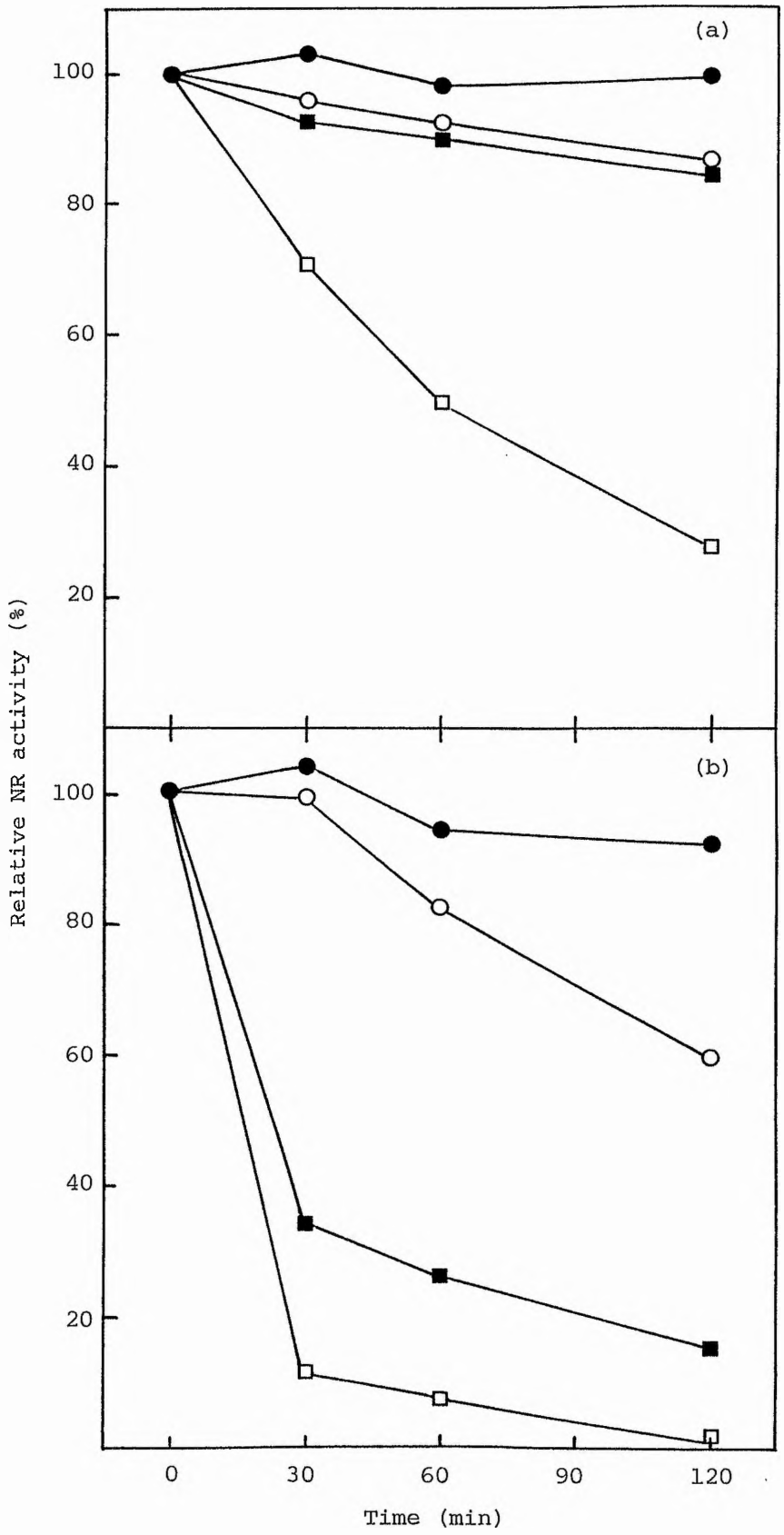
Effect of Temperature on the Ability of BSA to Stabilise NR in Extracts of 4 and 6 Day Old Primary Leaves

Earlier in this chapter it was shown that BSA was able to protect NR from age-dependent inactivation in cell free extracts at 4°C. Since Kuo buffer was also able to do this at 25°C the ability of BSA buffer to stabilise NR

Fig.17

Effect of Type of Extraction Buffer on the Stability of NR in Extracts of 4 and 8 Day Old Plants Maintained at 4°C and 25°C

4 day old primary leaves were extracted in Buffer I (■) or Kuo buffer (●) and 8 day old primary leaves were extracted in Buffer I (□) or Kuo buffer (○). After centrifugation of the brei at 38 000 g for 20 min the extracts, which contained 4.89, 2.90, 0.39 and 0.20µmol nitrite produced/ml extract/h respectively were maintained at 4°C (a) or 25°C (b) and aliquots removed at the times indicated for assay of NADH-NR activity.


(a)
(b)
100
80
60
40
20
Relative NR activity (%)
0
30
60
90
120
Time (min)

at this higher temperature was investigated (Fig.18).

As one might expect, NR from 4 day old primary leaf extract was more labile at 25°C than 4°C and the highly labile NR from 6 day old primary leaf extract was even less stable at this higher temperature. It is clear that BSA loses its ability to stabilise NR at 25°C. However, the mode of decay of NR activity in the control was quite different to that in the BSA treatment at this temperature. In addition, NR activity in both the 4 and 6 day old BSA leaf extracts decayed in a very similar fashion, unlike the situation without BSA (Fig.18b).

Kuo *et al* (1982a) also observed that the presence of non-plant protein (in their case casein) improved the stability of NR in phosphate buffer at 0°C but failed to do so at 30°C.

As the stability of NR in Kuo buffer extracts was only slightly affected by the elevated incubation temperature it would appear that the major stabilising effect of this buffer is quite different from that of BSA buffer. A specific parameter of the Kuo buffer was therefore investigated, namely buffer pH.

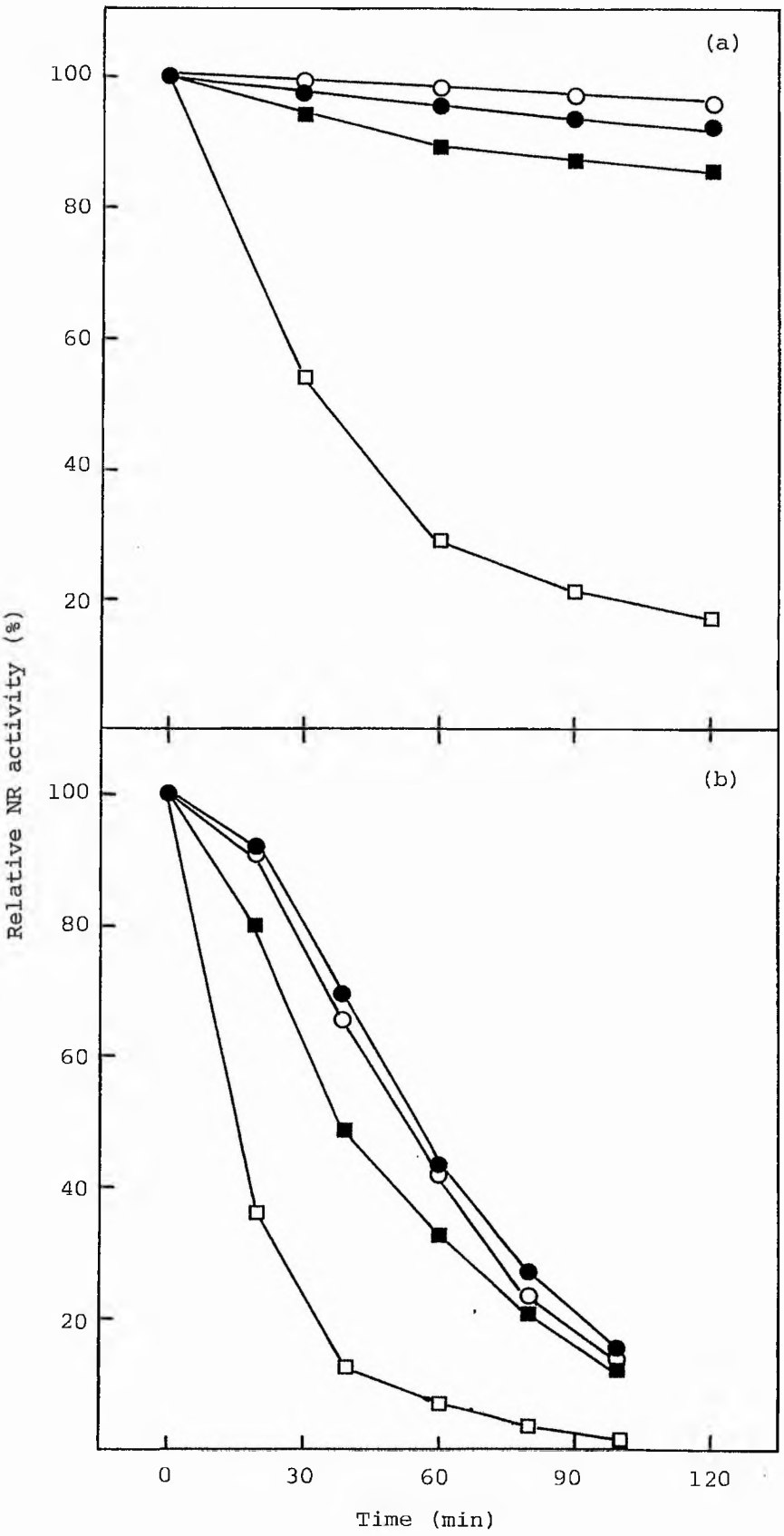
Effect of pH on the NR-Stabilising Ability of Buffer I and Kuo Buffer

Kuo (1979) found that NR activity was most stable in leaf tissue that had been extracted with buffer in the pH range 8.2 - 8.7. Since NR has optimal activity at

Fig.18

Effect of Presence of BSA on the Stability of NR in Extracts of 4 and 6 Day Old Plants Maintained at 4°C and 25°C

4 day old primary leaves were extracted in Buffer I containing no additions (■) or 3% BSA (●) and 6 day old primary leaves were extracted in Buffer I containing no additions (□) or 3% BSA (○). After centrifugation of the brei for 20 min at 38 000 g the extracts which contained 2.39, 2.50, 1.65 and 2.59µmol nitrite produced/ml extract/h respectively were maintained at 4°C (a) and 25°C (b) and aliquots removed at the times indicated for assay of NADH-NR activity.


(a)
(b)
100
80
60
40
20
Relative NR activity (%)
0
30
60
90
120
Time (min)

pH 7.5 it was suggested that the stabilising effect of pH could not be due to optimising pH for catalysis. However, since these authors assayed NR using buffer of pH 7.5 this statement does not automatically follow. They also suggested that the high pH might stabilise NR by inhibition of proteolytic enzymes in the extract.

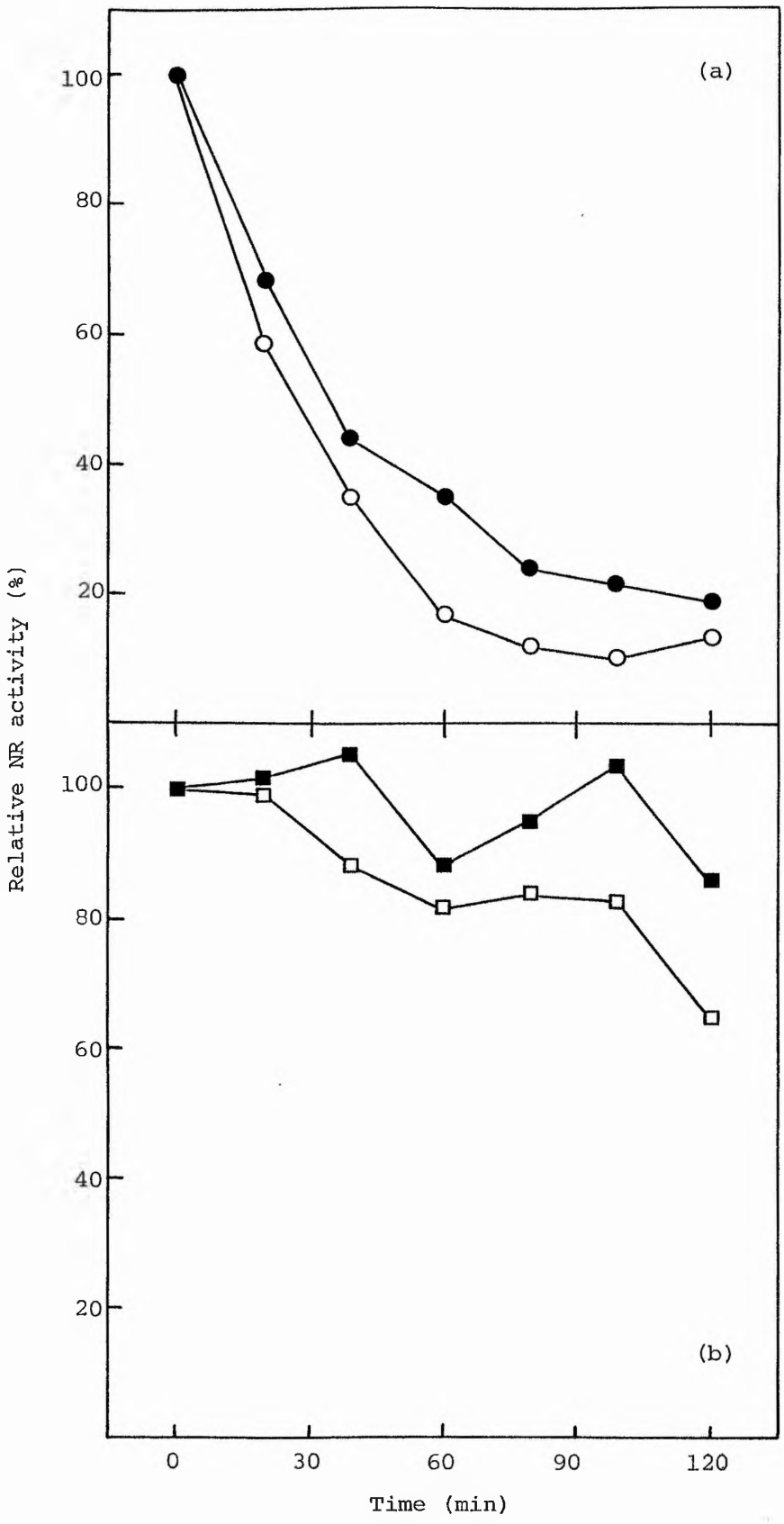
Loss of NR activity at 4°C was monitored in centrifuged extracts of 8 day old primary leaves extracted in Buffer I and Kuo buffer adjusted to pH 7.5 and 8.5 to assess the contribution of pH to stability of NR in crude extracts (Fig.19).

Reducing the pH of Kuo buffer from 8.5 to 7.5 resulted in a decrease in stability of NR while increasing the pH of Buffer I from 7.5 to 8.5 increased the stability of NR. However, increasing the pH of Buffer I to 8.5 did not increase the stability of NR to the level of that observed in Kuo buffer at pH 8.5 or pH 7.5. It is known that Tris, with a pk_a of 8.3 has a higher buffering capacity than phosphate at high pH but even taking this into account it is likely that pH is not the only factor involved in the superior stabilising ability of Kuo buffer on NR.

Fig.19

Effect of pH on the NR Stabilising Ability of Buffer I and Kuo Buffer

8 day old primary leaves were extracted in (a) Buffer I adjusted to pH 7.5 (○) or pH 8.5 (●) and (b) Kuo buffer adjusted to pH 7.5 (□) or pH 8.5 (■). After centrifugation of the brei at 38 000 g for 20 min the extracts, which contained 0.15, 0.28, 0.37 and 0.22µmol nitrite produced/ml extract/h respectively, were maintained at 4°C and aliquots removed at the times indicated for assay of NADH-NR activity.


(a)
(b)
Relative NR activity (%)
100
80
60
40
20
0
30
60
90
120
Time (min)

Effect of Kuo Buffer on the Conversion of NR to Smaller NADH-CR Species in Extracts of 8 Day Old Primary Leaves

To determine whether, like BSA, Kuo buffer stabilises NR by decreasing the conversion of NR to smaller NADH-CR species Kuo buffer extracts were subjected to sucrose density gradient analysis (Fig.20).

One cannot say that Kuo buffer prevents the conversion of NR to smaller NADH-CR species since the level of small NADH-CR species in the extract (Fig.20c) is greater than that observed under any other conditions investigated during this work, especially when the NR complex was apparently stable. It is likely that the small NADH-CR species in the Buffer I extracts do not accumulate once converted as they in turn are susceptible to action of proteinases. Whether these species represent *in vitro* breakdown products or whether they represent species present *in vivo*, it is possible that they may also be stabilised by Kuo buffer since it has been suggested that the high pH of the buffer may stabilise enzyme activity by inhibition of proteinases.

Continued presence of Kuo buffer was found to be necessary for stabilisation of NR since the NR in Kuo buffer extracts broke down on gradients prepared in phosphate buffer (Fig.20b).

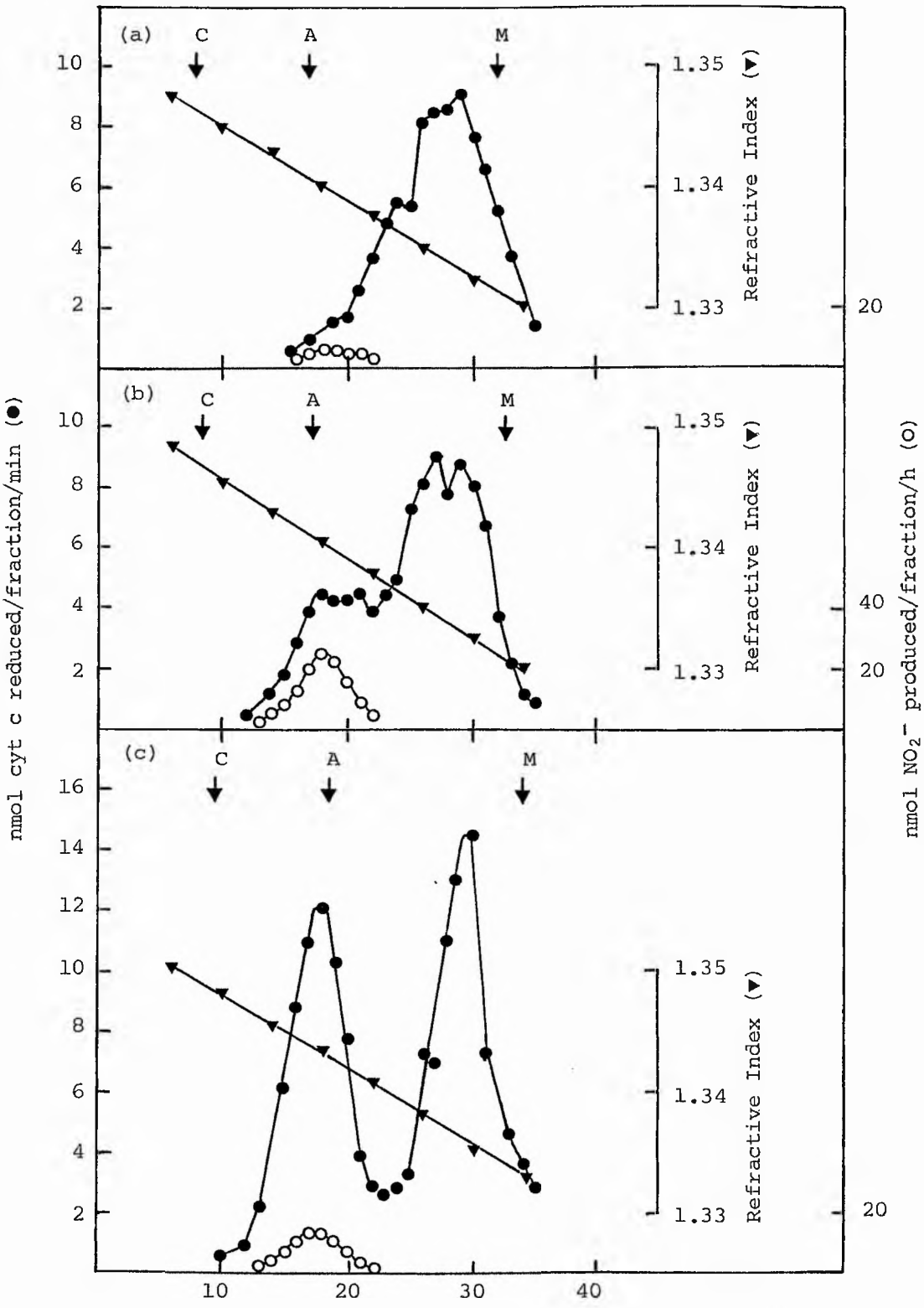
Attention is drawn to the apparently low level of NR activity associated with the 7.7S NADH-CR species, the intact NR complex. If, as has previously been contended,

Fig.20

Sucrose Density Gradient Centrifugation Analysis of 8 Day Old Primary Leaf Extracts Prepared with Buffer I or Kuo Buffer

8 day old primary leaves were extracted in Buffer I or Kuo buffer (3 ml buffer/g FW tissue) and the brei centrifuged at 38 000 g for 20 min.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after a 0.4 ml aliquot of extract prepared with (a) Buffer I was analysed on a gradient prepared with phosphate buffer (b) Kuo buffer was analysed on a gradient prepared with phosphate buffer and (c) Kuo buffer was analysed on a gradient prepared with Kuo buffer. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
(b)
(c)
C
A
M
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)
Refractive Index (▼)
1.35
1.34
1.33
10
8
6
4
2
16
14
12
20
40
30

this is due to a salt effect which renders the enzyme less active then it would appear that the dehydrogenase moiety of the complex is spared since the NADH-CR activity of the 7.7S NADH-CR species is apparently not affected.

CHAPTER 3

PURIFICATION OF BARLEY LEAF NITRATE REDUCTASE

Purification of Barley Nitrate Reductase

Plants no older than 90 h were used for NR purification since they were found to yield the greatest amount of the most stable NR activity after extraction. In routine NR preparations 200 - 300 g barley shoots were extracted and purified by $(NH_4)_2SO_4$ fractionation, large-scale gel filtration and BDS affinity chromatography according to the procedure described in Methods, Section II. A typical example of this procedure is discussed below.

Unfortunately it was not possible to remove green-coloured and high molecular weight material during the first centrifugation step of the purification procedure i.e. preparation of a cell-free extract due to lack of rotor capacity for centrifugation at high speed.

It was found by Small (1980) that the bulk of NR in 90 h old barley seedling extract precipitated between 30 and 45% $(NH_4)_2SO_4$ saturation. While 30% $(NH_4)_2SO_4$ saturation precipitated most of the green coloured material and the high molecular weight constitutive NADH-CR species, it also precipitated approximately 20-35% of the NR activity. This was considered to be an unacceptable loss of activity and 0-45% $(NH_4)_2SO_4$ fractionation was employed as the first stage in the purification procedure and high molecular weight material removed at the next stage. The small, 27 800 MW NADH-CR species which has been shown to be unrelated to NR was however removed at this stage (Small, 1980).

The sample was then subjected to large-scale gel filtration chromatography on Biogel A 1.5 m (Fig.21). Green material eluted as a band at the void volume which was allowed to travel almost to the bottom of the column before collection of fractions commenced. A substantial amount of NR activity eluted at the void volume along with the green-coloured and high molecular weight material. This is likely to be due to non-specific association of NR rather than the existence of high molecular weight forms of NR. In order to reduce the amount of NR activity eluting with the void volume an attempt was made to remove the high molecular weight material before Biogel chromatography. To do this the $(NH_4)_2SO_4$ fraction was resuspended in a greater than minimum volume of Buffer II (usually 100 ml) to ensure that it was completely dissolved and centrifuged at 100 000 g for 20 min. This removed all but a trace of the green components, presumably along with the constitutive high molecular weight NADH-CR species, while retaining virtually all of the NR activity (98.5%). The supernatant (containing 715 mg protein) was concentrated by $(NH_4)_2SO_4$ fractionation before application to the Biogel A 1.5 m column (Fig.22).

NR activity still eluted with the void volume but it was proportionally less than when the additional centrifugation was omitted. This modified procedure did not result in a greater yield of NR at this stage. This was partly due to loss of activity during the $(NH_4)_2SO_4$ concentration step since little loss of NR activity was

Fig.21

Biogel A 1.5 m Gel Filtration of the 0-40% $(NH_4)_2SO_4$ Fraction Derived from 225 g 4 Day Old Barley Shoots

20 ml of the $(NH_4)_2SO_4$ fraction containing 1 866 mg protein were applied to a Biogel A 1.5 m column (4.3 x 87 cm). NR was eluted with Buffer II and 13 ml fractions were collected and assayed for NADH-NR activity. Elution volume is expressed as volume after the void volume to allow comparison with Fig.22.

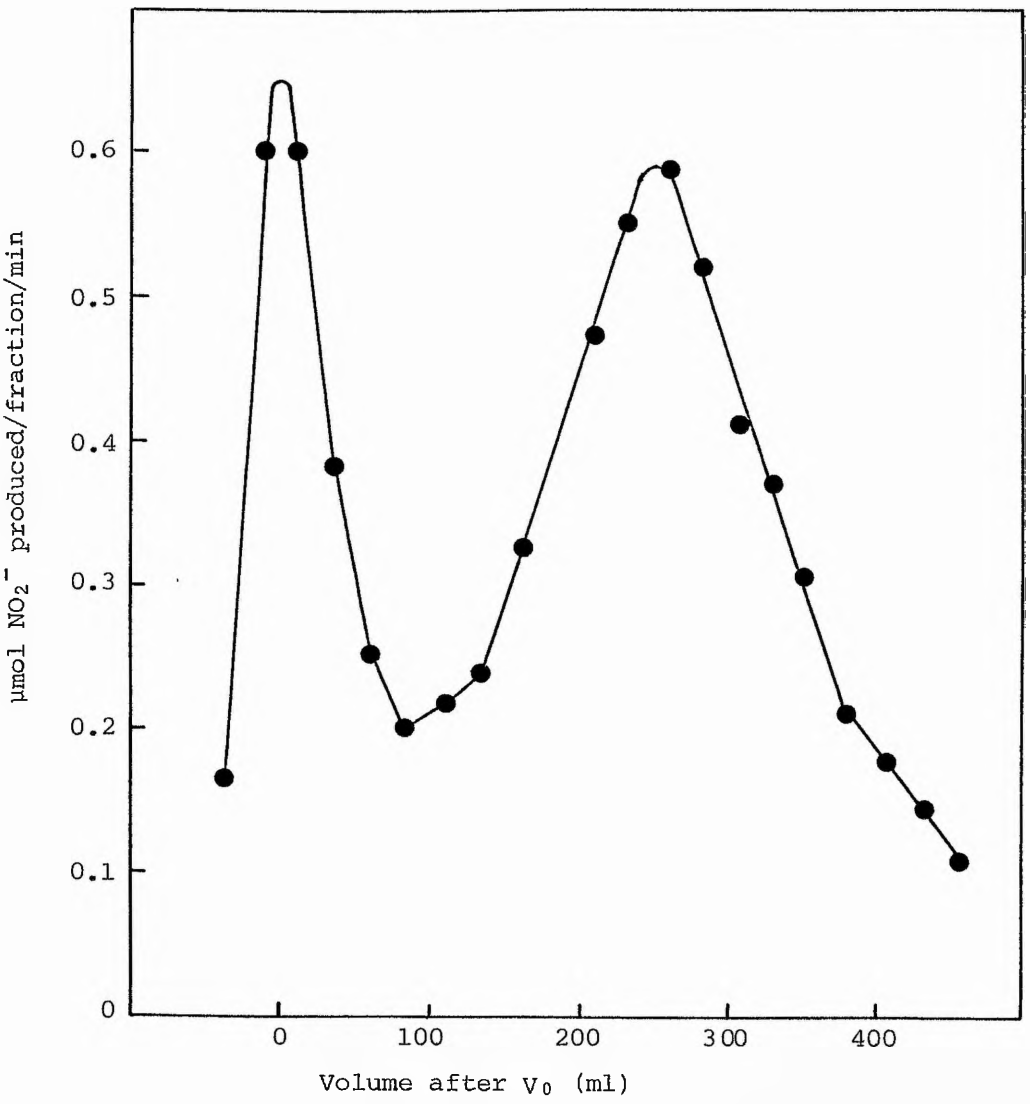
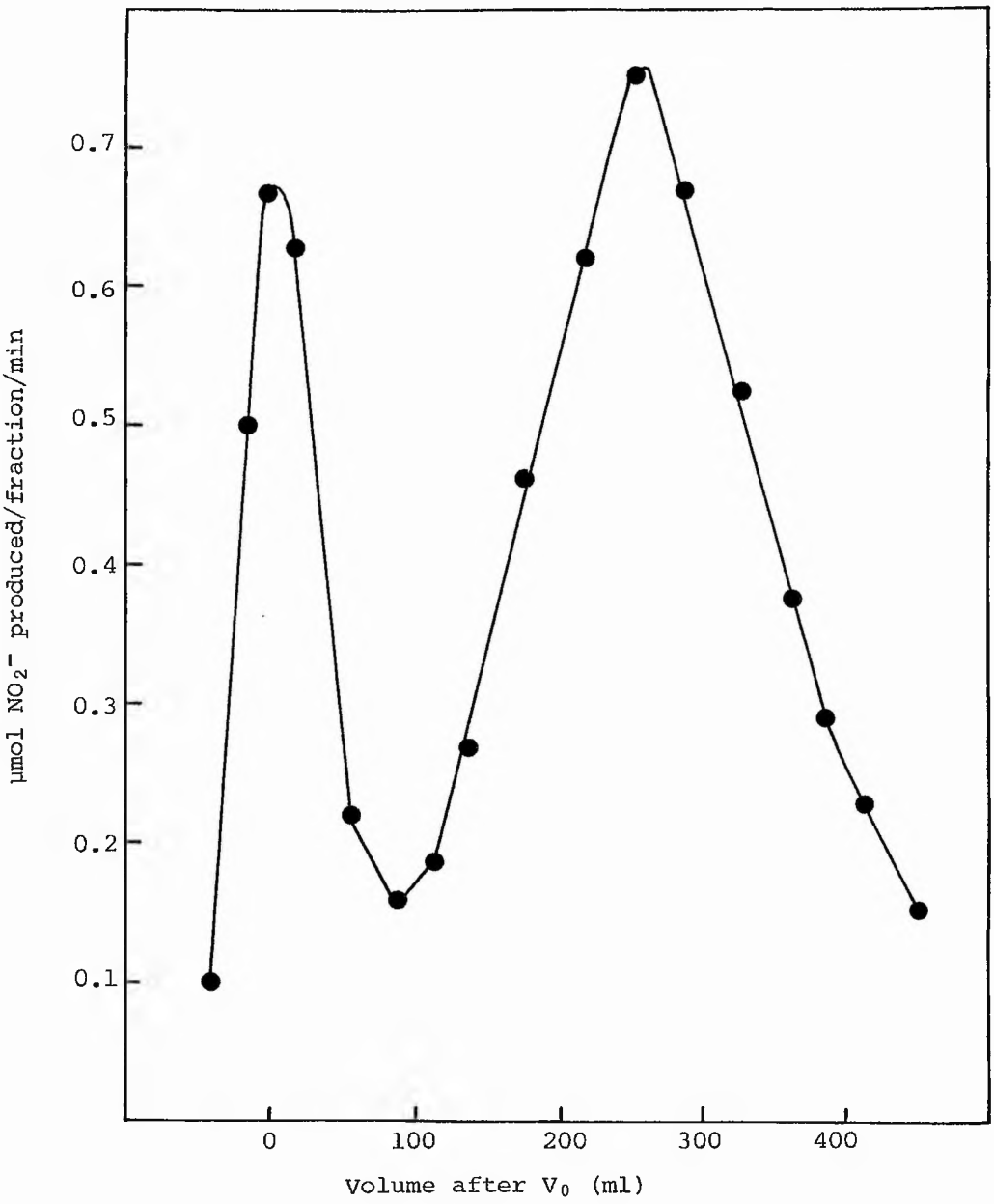

µmol NO_2^- produced/fraction/min
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
Volume after V_0 (ml)

Fig.22

Biogel A 1.5 m Gel Filtration of the 0-40% $(NH_4)_2SO_4$ Fraction Derived from 285 g 4 Day Old Barley Shoots After Centrifugation at 100 000 g

20 ml of the 100 000 g supernatant containing 715 mg protein were applied to a Biogel A 1.5 m column (4.3 x 87 cm). NR was eluted with Buffer II and 19.5 ml fractions were collected and assayed for NADH-NR activity. Elution volume is expressed as volume after the void volume to allow comparison with Fig.21.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
µmol NO2- produced/fraction/min
0
100
200
300
400
Volume after V0 (ml)

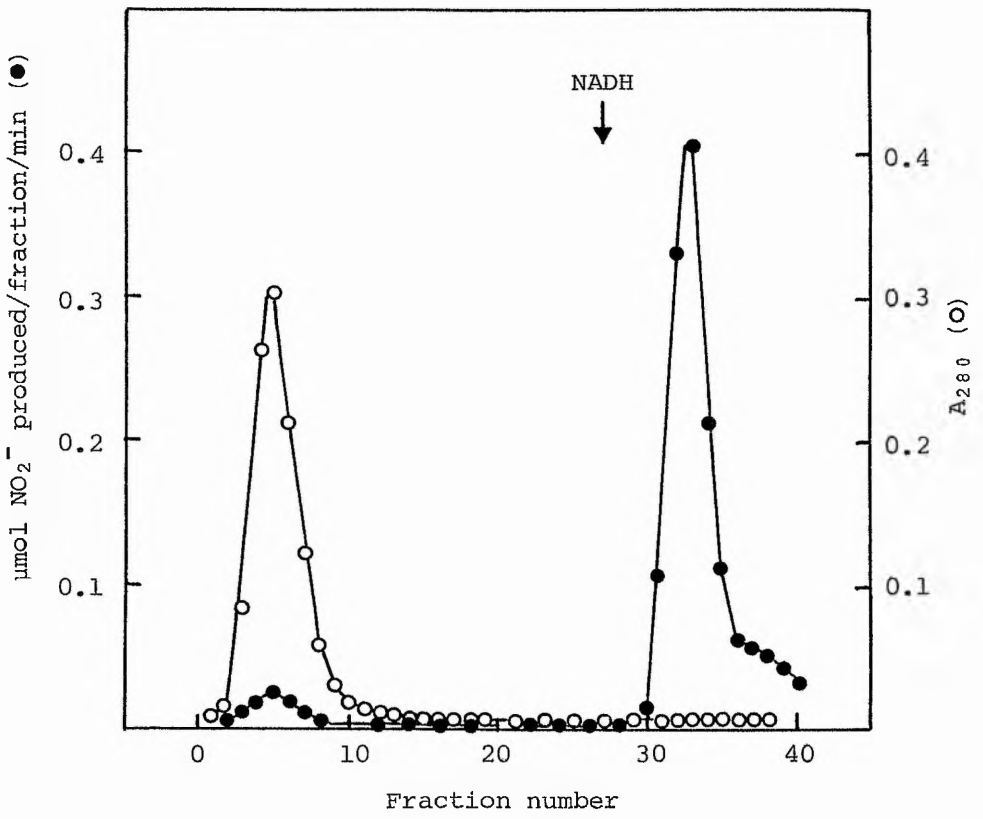
observed immediately after centrifugation. However, it did result in an approximate two-fold increase in specific activity after Biogel A 1.5 m chromatography and also provided the potential for larger samples to be processed since protein applied to the column was halved.

Further purification was achieved by affinity chromatography on Blue Dextran Sepharose using samples which had been passed through Biogel A 1.5 m and stored for up to 3 months as previously described in Methods (Fig.23). A small amount of NR (2% of the total NR eluted) did not bind to Blue Dextran Sepharose and passed through the column with the majority of applied protein (typically 40 - 100 mg) while 5μM NADH eluted NR in a discrete peak with negligible protein. NR yields from Blue Dextran Sepharose chromatography were consistently low (10-30%) and 0.3M KNO_3 (which functions by raising the ionic strength of the elution buffer) was required to elute the remaining NR bound to the column (data not shown). This would appear to be a commonly observed phenomenon and Small (1980) concluded that NADH elutes the NR which is electrostatically bound to the column via the NADH-binding site of the enzyme whereas KNO_3 can elute all electrostatically bound enzyme, regardless of binding sites. Poule and Oaks (1983) have recently noted the existence of two interconvertible forms of corn leaf NR eluted from Blue Dextran Sepharose. NR eluted with 10μM NADH had a MW of 200 000 while that eluted with 0.3M KNO_3 or 0.3M KCl had a MW of 433 000.

Fig.23

Elution of NR from Blue Dextran Sepharose

The sample which had been passed through Biogel A 1.5 m and stored, in this case, for 9 days in 40% glycerol at -70°C was thawed at room temperature and glycerol removed from the sample by precipitating protein with an equal volume of saturated $(NH_4)_2SO_4$ solution pH 7.5. The protein was collected by centrifugation and redissolved in 10 ml of Buffer III (75 mg protein). The sample was applied to a Blue Dextran Sepharose column (1.8 x 8.5 cm) pre-equilibrated with Buffer III. Non absorbed protein (o) was eluted by Buffer III, NADH-NR (•) was eluted by Buffer III containing 5µm NADH and 6 ml fractions were collected.


NADH
0.4
0.3
0.2
0.1
µmol NO_2^- produced/fraction/min (●)
A_{280} (○)
0
10
20
30
40
Fraction number

This phenomenon was not investigated in the system used here.

The specific activity of the NR purified by the method described here was low (1.31 units/mg) and because the purified NR was highly unstable, losing 50% of its activity during a 1 h incubation on ice, specific activity decreased dramatically with time therefore NR concentrated against PEG 6 000 at 4°C had a much lower specific activity (often as low as 0.2 units/mg).

Most specific activities reported for Blue Dextran Sepharose-purified higher plant NR have been below 10 units/mg e.g. Kuo *et al* (1980) obtained a specific activity of 8.00 units/mg using Barley cv. Steptoe. There are however notable exceptions such as the specific activity of 24.12 units/mg achieved by Notton *et al* (1977) using spinach.

It can be seen from Tables 15 and 16 that major losses of NR were incurred before the preparation was subjected to Blue Dextran Sepharose chromatography and attempts have subsequently been made in this laboratory to stabilise NR during purification (J.McA. Campbell and Wray, 1983) using improved buffer systems, inclusion of proteinase inhibitors and improved plant growth procedures. The results of these studies will be analysed in the General Discussion with particular reference to the results obtained in Chapter 2 of the Results.

Table 15 Purification of NR from 285 g 4 Day Old Barley Shoots

Purification Step	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification (fold)
Filtered extract	729	5766	20.02	0.0035	100	1
38 000 g spin supernatant	713	3028	17.66	0.0058	88.2	1.66
0-45% $(NH_4)_2SO_4$ fraction	100	1436	15.81	0.0110	79.0	3.14
100 000 g spin supernatant	96	806	15.58	0.019	77.8	5.52
0-45% $(NH_4)_2SO_4$ fraction	20	715	13.51	0.019	67.5	5.52
Pooled Biogel A 1.5 m peak	274	84	7.60	0.091	38.0	25.97
0-45% $(NH_4)_2SO_4$ fraction	14	79	6.87	0.087	34.3	24.83
0-50% $(NH_4)_2SO_4$ fraction of extract stored for 9 days	10	75	6.35	0.084	31.7	24.00
Pooled BDS peak	30	0.59	0.77	1.305	3.8	372.86

NR units: 1 unit is defined as 1µmol nitrite formed/min at 25°C.

Table 16 Purification of NR from 225 g 4 Day Old Barley Plants up to Biogel A 1.5 m Chromatography, Omitting Centrifugation at 100 000 g

Purification Step	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (units(mg)	Yield (%)	Purification (fold)
Filtered extract	690	5506	20.66	0.0038	100	1
38 000 g spin supernatant	669	3519	19.23	0.0055	93.1	1.45
0-45% $(NH_4)_2SO_4$ fraction	25	1866	13.50	0.0072	65.3	1.89
Pooled Biogel A 1.5 m peak	270	164	6.79	0.0414	32.9	10.89
0-45% $(NH_4)_2SO_4$ fraction	76	132	5.85	0.0443	28.3	11.66

Assessment of Purity of the Purified NR Preparation by Polyacrylamide Gel Electrophoresis Under Non-Denaturing Conditions in 5% Polyacrylamide Gels

50µl samples of PEG 6 000-concentrated Blue Dextran Sepharose purified NR containing approximately 5-10µg of protein were subjected to non-denaturing gel electrophoresis in 5% acrylamide gels as described in Methods, Section V and gels stained for protein, MVH-NR activity and NBT reductase activity.

No protein staining bands were detected as insufficient protein was applied to the gels making assessment of purity of the NR preparation impossible. It was not possible to apply more protein per gel using the method employed for concentrating the sample and alternatives (such as an Amicon concentrator) were not available at the time. Staining for MVH-NR activity resulted in a broad achromic band with an R_f of 0.25 ± 0.02 (6 gels). In contrast to the findings of Small (1980) who detected two bands of NBT reductase activity with R_f values of 0.23 and 0.29, multiple bands of activity were observed here. The major band comigrated with MVH-NR activity at 0.24 ± 0.01 (6 gels). Since it was not possible to assess the purity of the enzyme preparation used it is not known whether these multiple bands represent proteins with NBT reductase activity or whether they were due, perhaps in part, to the so-called "no-dehydrogenase"-phenomenon associated with NBT stains (J. Doran, personal communication).

J.McA. Campbell *et al* (1983) have subsequently shown there to be a single protein species in NR samples prepared in a similar way to that described here which comigrated at R_f 0.23 with MVH-NR activity and a single band of NBT-reductase activity.

SDS Polyacrylamide Gel Electrophoresis of Purified Barley NR in 7.5% Acrylamide Gels

Aliquots of PEG 6 000-concentrated, Blue Dextran Sepharose-purified NR were subjected to SDS gel electrophoresis using a modified Laemmli discontinuous buffer system as described in Methods, Section V. Aliquots of a solution containing the standard proteins phosphorylase b (93 500 MW), BSA (68 000 MW), ovalbumin (45 000 MW) and myoglobin (17 200 MW) were run separately in parallel gels to the NR samples. The calibration plot derived from the molecular weight standards is presented in Fig.24.

Several protein bands were detected in the NR preparation including a major one of 61 000 MW, minor bands of MW 107 000, 78 000, 50 000 and 41 000 and faint bands of MW 67 000, 55 000 and 34 000.

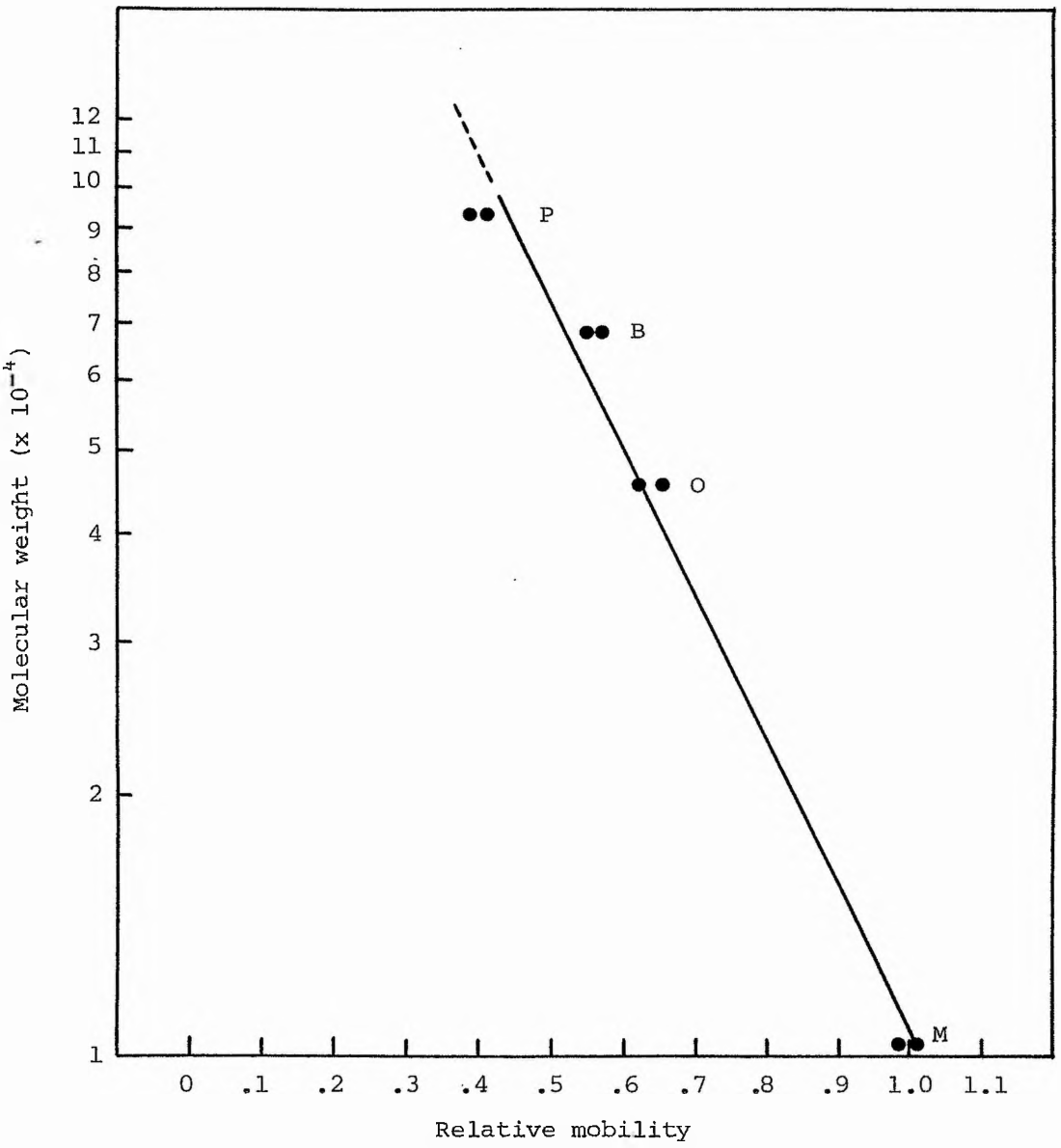
Why protein staining was possible on SDS gels but not non-denaturing gels when approximately equivalent amount of protein might be expected to be present is not known but the phenomenon was also observed by Small (1980). It has, however, been suggested by B.A. Notton (personal communication) that native NR aggregates and is thus unable to enter the polyacrylamide gel while the subunit can.

Fig.24

Molecular Weight Calibration Plot for SDS Gel Electrophoresis

This figure shows the relationship between the relative mobility of protein standards in SDS gels and their molecular weight. Letters refer to protein standards as follows: P, phosphorylase b (92 500 MW); B, BSA (68 000 MW); O, ovalbumin (45 000 MW); M, myoglobin (17 200 MW).

Electrophoresis was carried out as described in Methods, Section V.


Molecular weight (x 10^{-4})
12
11
10
9
8
7
6
5
4
3
2
1
P
B
O
M
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
Relative mobility

Since it was not possible to assess the purity of the purified NR preparation, interpretation of these results is made difficult. Analysis of the data with reference to data from other systems will be attempted in the General Discussion where models for the structure of higher plant NR will also be discussed.

Alternative Methods for the Purification of Barley Leaf NR

There have been reports in the literature of rapid purification of higher plant NR using essentially two-step procedures involving fractionation with $(NH_4)_2SO_4$ followed immediately by Blue Dextran Sepharose chromatography.

Sherrard and Dalling (1979) using only 10-15 g of wheat leaf tissue obtained a 2 573-fold purification of NR with a final specific activity of 23.1 units/mg using a procedure involving: extraction with 25mM potassium phosphate buffer pH 7.0 containing 5mM EDTA, 5mM cysteine; centrifugation at 25 000 g for 15 min; fractionation with 30-45% $(NH_4)_2SO_4$ followed by Blue Dextran Sepharose chromatography on a column 2.3 x 8.0 cm and elution with 5µM NADH.

Kuo *et al* (1980) achieved a 573-fold purification of barley cv. Steptoe leaf NR using 25-30 g of tissue with a specific activity of 8.00 units/mg. Tissue was extracted with 0.025M Tris HCl, 1mM DDT, 1mM EDTA, 1µM FAD, 1µM sodium molybdate and 3% casein, pH 8.2; centrifuged for 30 min at 40 000 g; fractionated with

50% $(NH_4)_2SO_4$ and loaded onto a Blue Dextran Sepharose column (2.6 x 10 cm) pre-equilibrated with extraction buffer lacking casein (Buffer A). NR activity was eluted with a 0-280μM linear gradient of NADH after washing with 2 bed volumes of buffer A, 3 bed volumes of Buffer A containing 0.3M Tris HCl and re-equilibrating with Buffer A.

The advantages of a rapid purification procedure for a highly labile enzyme are obvious, therefore preliminary attempts were made to purify NR essentially by the methods of Sherrard and Dalling and Kuo *et al* described above.

The NR elution profiles from Blue Dextran Sepharose chromatography of NR samples derived by modifications of the procedures of Sherrard and Dalling and Kuo *et al* are presented in Fig.25 and Fig.26 respectively. The purifications achieved using the two methods are presented in Table 17a and Table 17b respectively.

As can be seen from the purification procedures described in the legend to Figs.25 and 26 the original methods were not strictly adhered to. In addition it was not possible to measure the protein concentrations of the Blue Dextran Sepharose fractions, even when the samples were concentrated, with any degree of accuracy. Final specific activities could not, therefore, be compared but it is clear by comparing NR activities during the various stages that purification was not as successful for either

Fig.25

Blue Dextran Sepharose Chromatography of the 30-45% $(NH_4)_2SO_4$ Fraction Prepared Essentially as Described by Sherrard and Dalling (1979)

15 g of 4 day old primary leaves were cut into small pieces and ground in a mortar and pestle with Buffer I (1 g FW:3 ml buffer) and the homogenate was centrifuged at 38 000 g for 15 min. Protein fractionating between 30 and 45% $(NH_4)_2SO_4$ saturation was dissolved in ice cold Buffer I so that the final volume was $\frac{1}{4}$ of the volume of the crude extract and then loaded onto a Blue Dextran Sepharose column (1.8 x 8.5 cm) pre-equilibrated with Buffer III. The column was then washed with 100 ml Buffer III and NR eluted from the gel by 5µM NADH in Buffer III. The flow rate during loading, washing and elution was approximately 2 ml/min and 4.25 ml fractions were collected.

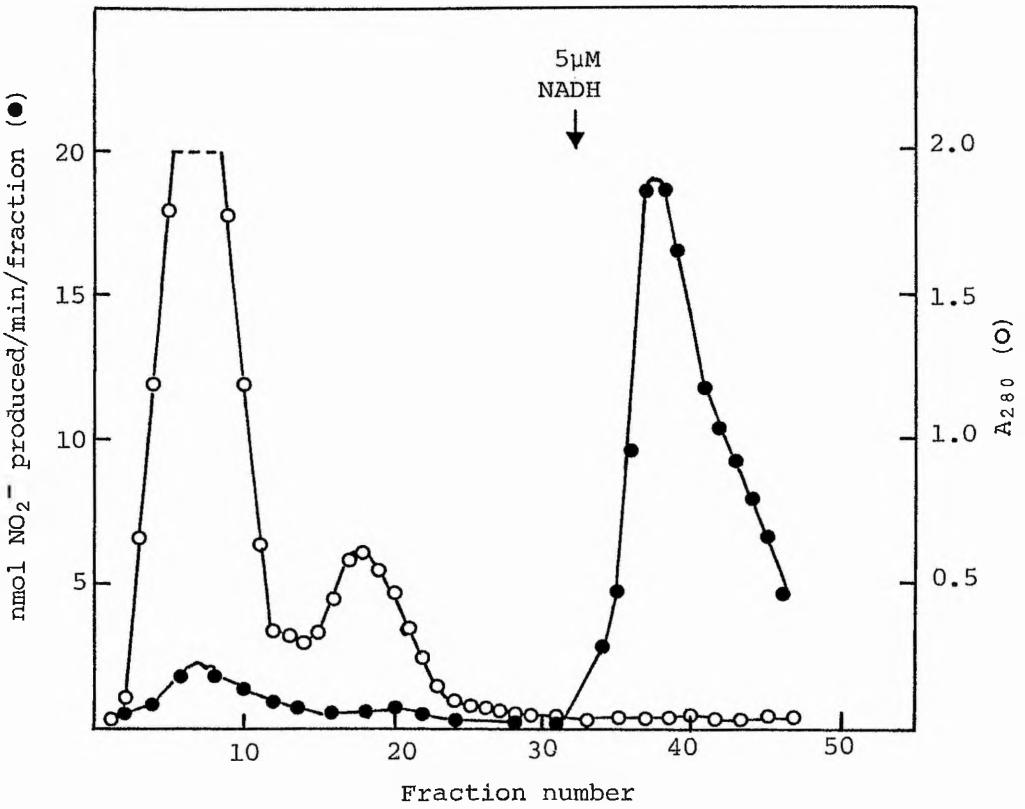

5μM
NADH
nmol NO_2^- produced/min/fraction (●)
A_{280} (○)
Fraction number
20
15
10
5
2.0
1.5
1.0
0.5
10
20
30
40
50

Fig.26

Blue Dextran Sepharose Chromatography of the 0-50% $(NH_4)_2SO_4$ Fraction Prepared Essentially as Described by Kuo *et al* (1980)

26 g of 4 day old primary leaves were ground in a mortar and pestle with 0.025M Tris HCl, 1mM DTT, 1mM EDTA, 1µM FAD, 1µM sodium molybdate, pH 8.2 (Buffer A) and centrifuged for 30 min at 100 000 g. (Kuo *et al* centrifuged the extract at 40 000 g but it was thought preferable to remove high MW and green coloured material at this stage). The supernatant was adjusted to 50% saturation with respect to $(NH_4)_2SO_4$ using an equal volume of saturated $(NH_4)_2SO_4$ in 0.025M Tris HCl, pH 8.2. Precipitated proteins were collected by centrifugation and redissolved with Buffer A in the same volume as the initial crude extract. The sample was loaded onto a column (1.8 x 8.5 cm) pre-equilibrated with Buffer A. The column was then washed with Buffer A until the A_{280} was reduced to less than 0.05, followed by three column volumes of 0.25M Tris HCl pH 8.2 containing the same additions as Buffer A. NR activity was then eluted with 5µM NADH. 6 ml fractions were collected throughout.

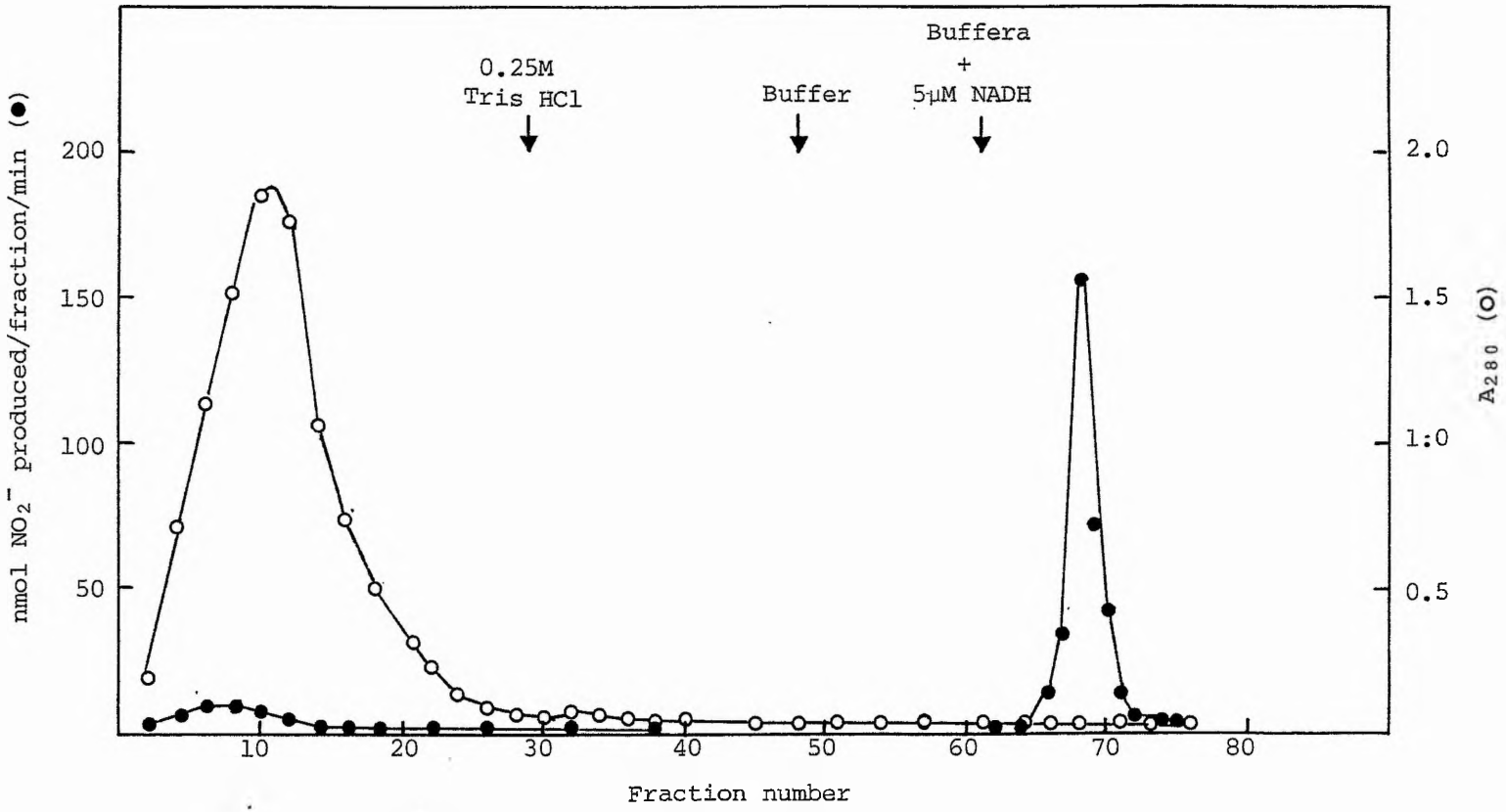

0.25M
Tris HCl
Buffer
Buffera
+
5μM NADH
nmol NO_2^- produced/fraction/min (●)
A_{280} (○)
Fraction number
200
150
100
50
2.0
1.5
1:0
0.5
10
20
30
40
50
60
70
80

Table 17 Alternative Methods for the Purification of Barley NR

Purification Step	Activity (units)	Protein (mg)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
(A) After Sherrard & Dalling (1979)					
Cell free extract from 15 g of barley leaves	0.930 (1.427)	165 (158.5)	0.006 (0.009)	1 (1)	100 (100)
30-45% $(NH_4)_2SO_4$ fraction	0.674 (0.896)	36 (30.9)	0.019 (0.029)	3.2 (3.2)	72 (63)
Pooled BDS peak	0.131 (0.301)	(0.01)	(23.1)	(2573)	14 (21)
(B) After Kuo *et al* (1980)					
Cell free extract from 26 g of barley leaves	1.55 (5.85)	286 (411)	0.005 (0.014)	1 (1)	100 (100)
0-50% $(NH_4)_2SO_4$ fraction	1.41 (4.40)	143	0.01	2	66 (75)
Pooled BDS peak	0.33 (3.12)	(0.39)	(8.00)	(573)	21 (53)

Figures in brackets denote values obtained by Sherrard and Dalling and Kuo *et al* for NR purification from similar quantities of wheat and barley leaves (see text for details)

method used. However, using the additional NR stabilising procedures described by J.McA. Campbell and Wray (1983) it would appear that a two step procedure for purification of NR is possible. It is interesting to note that the Kuo *et al* method was able to recover 0.39 mg NR from 25-30 g tissue while Sherrard and Dalling using 'conventional' methods of stabilising NR only recovered 0.01 mg NR from 15 g tissue.

CHAPTER 4

PURIFICATION OF AN NADH-CYTOCHROME *c* REDUCTASE SPECIES POSTULATED TO BE DERIVED FROM BARLEY NITRATE REDUCTASE

Purification of an NADH-CR Species Postulated to be Derived from NR

It has been shown in a previous part of the Results (Chapter 2) that as the barley leaf ages the extractable NR activity decreases and the level of small NADH-CR species increases. Some of these species have been shown by Small (1980) and Small and Wray (1980a) to possibly be derived from NR. It was indicated that the most predominant small NADH-CR species found in cell-free extracts of 7 day old barley leaves (the 3.1S species) was also likely to be the most stable and therefore an ideal species to isolate and purify. The 3.1S NADH-CR species was found to bind to Blue Dextran Sepharose, which is not surprising since it uses NADH as reductant and presumably therefore possesses a dinucleotide fold (Thompson *et al*, 1975). This small NADH-CR species eluted from Blue Dextran Sepharose under the same conditions as for NR. Small (1980) succeeded in purifying this species but was unable to characterise it. The aim of the work described there was to characterise the NR-related NADH-CR species and to raise antibodies against it (see Results, Chapter 7).

Plants no younger than 6 days old were used for purification of the small NADH-CR species initially. A considerable variation in the stability of NR was encountered in extracts from seedlings derived from different batches of seeds. It was found necessary, in some cases, to grow barley seedlings for at least 10 days

to obtain a suitable proportion of small NADH-CR species compared to intact NR in the extract.

Although extracts of secondary leaves of 6 day old plants have been shown to possess more stable NR than extracts of primary leaves of 6 day old plants (Results, Chapter 2), inclusion of the secondary leaves in the harvest reduced the processing time and providing they were greater than 3-4 cm long were found to contribute negligible 'stable' NR to the extract (data not shown). Since 6 day old shoots were much more fibrous than 4 day old shoots, inclusion of a little acid-washed sand during grinding facilitated extraction and hence reduced extraction time.

Routinely 80-250 g of 6-10 day old barley shoots were purified by $(NH_4)_2SO_4$ fractionation, large-scale gel filtration and Blue Dextran Sepharose affinity chromatography according to the procedure described in Methods, Section II.

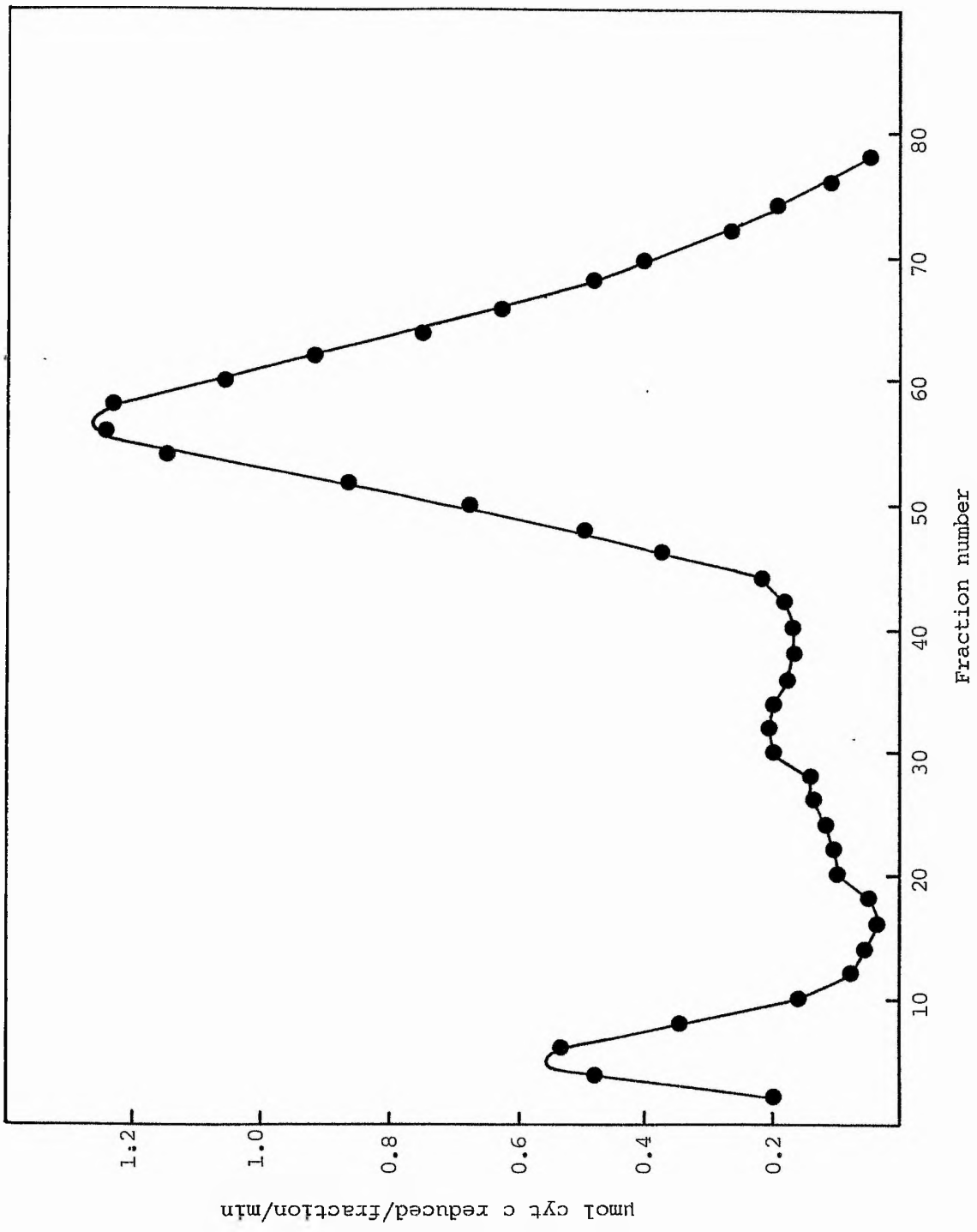
$(NH_4)_2SO_4$ fractionation (30-60%) removed most of the green coloured material and removed both the small and large constitutive NADH-CR species present in crude extracts (Small, 1980).

The $(NH_4)_2SO_4$ fractionate (maximum volume 20 ml containing 9-12 mg protein/g FW tissue) was then subjected to Biogel A 1.5 m gel filtration (Fig.27). Remaining traces of green material eluted as a band at the void volume which was allowed to travel almost to the bottom of the column before collection of fractions commenced. The major peak of NADH-CR activity eluted from the column

Fig.27

Biogel A 1.5 m Gel Filtration of the 30-60% $(NH_4)_2SO_4$ Fraction Derived from 80 g of 6 Day Old Barley Shoots

20 ml of the $(NH_4)_2SO_4$ fraction containing 832 mg of protein were applied to a Biogel A 1.5 m column (4.3 x 87 cm). NADH-CR activity was eluted with Buffer II and 9.3 ml fractions were collected.


μmol cyt c reduced/fraction/min
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
80
Fraction number

appeared to be symetrical. However, to avoid contamination by other NADH-CR species only the peak enzyme containing fractions were pooled.

Blue Dextran Sepharose chromatography was employed as the final stage in the purification procedure and as previously described the small NADH-CR species eluted under the same conditions as for NR (Fig.28). Usually all of the applied NADH-CR activity bound to the column, none passing through with the majority of applied protein (up to 100 mg) while 5μM NADH eluted NADH-CR activity immediately in a discrete peak with negligible protein.

It was found that the recovery of NADH-CR activity (due to the small NADH-CR species) was consistently higher (20-50%) than NR activity (10-30%) although still disappointingly low.

Details of the purification achieved are presented in Table 18.

Since there is more than one species of NADH-CR found in cell-free extracts of barley leaves, the value calculated for the initial specific activity of this purification is high as it is not specific for the 3.1S NADH-CR species. The 509-fold purification achieved therefore is likely to be under-estimated.

Fig.28

Elution of the 3.1S NADH-CR Species from Blue Dextran Sepharose

The sample which had been passed through Biogel A 1.5 m and stored for 6 days in 40% glycerol at -70°C was thawed at room temperature and glycerol removed from the sample by 0-67% $(NH_4)_2SO_4$ fractionation using twice the sample volume of saturated $(NH_4)_2SO_4$ solution, pH 7.5. The protein was collected by centrifugation and redissolved in 10 ml of Buffer III (35 mg protein). The sample was applied to a Blue Dextran Sepharose column (1.8 x 8.5 cm) pre-equilibrated with Buffer III.

Non-absorbed protein (o) was eluted with Buffer III, NADH-CR (•) was eluted with Buffer III containing 5μM NADH and 6 ml fractions were collected.

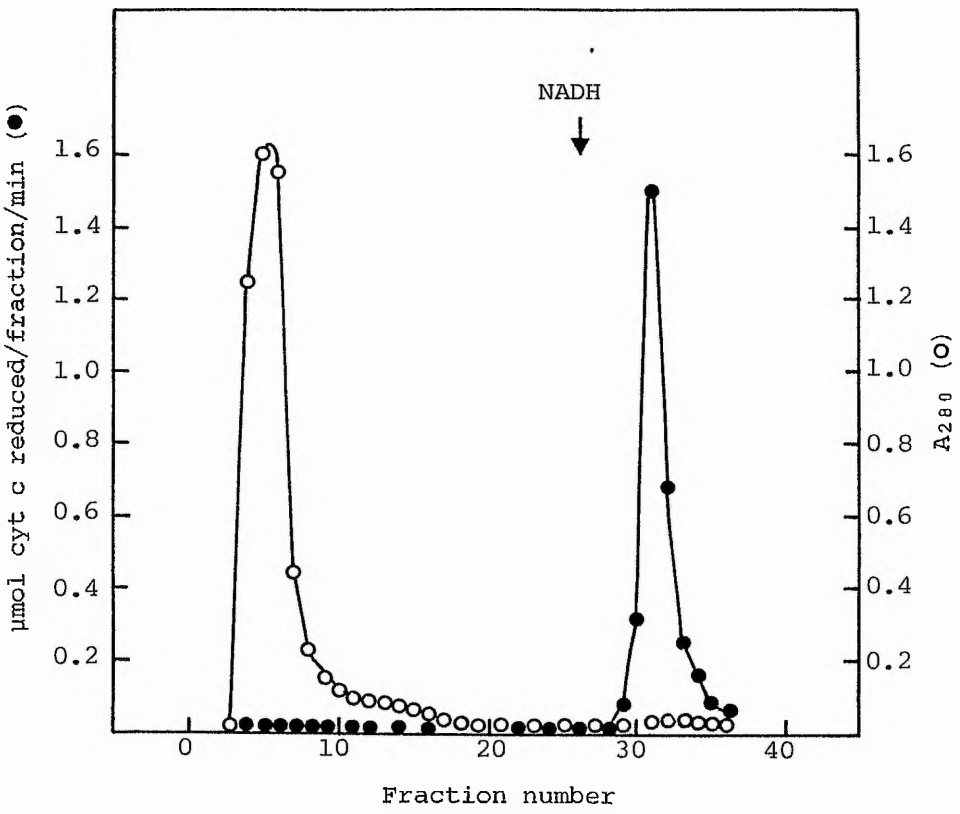

NADH
µmol cyt c reduced/fraction/min (●)
A280 (○)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
Fraction number

Table 18 Purification of the 3.1S NADH-CR Species from 80 g 6 day old Barley Shoots

Purification Step	Volume (ml)	Protein (mg)	Activity *(units)	Specific Activity (units/mg)	Yield (%)	Purification (fold)
38 000 g spin supernatant	250	1180	28.27	0.024	100	1
30-60% $(NH_4)_2SO_4$ fraction	135	832	21.63	0.026	76.5	1.08
Pooled Biogel A 1.5 m peak	186	82	5.94	0.072	21.0	3.00
Pooled Blue Dextran Sepharose peak	22.9	0.24	2.94	12.25	10.4	508.75

*1 unit is defined as 1µmol cytochrome c reduced/min

Assessment of Purity of the Preparation of the 3.1S NADH-CR Species

50µl samples of PEG 6 000-concentrated Blue Dextran Sepharose-purified 3.1S NADH-CR species, containing approximately 5µg protein were subjected to non-denaturing gel electrophoresis in 5% acrylamide gels as described in Methods, Section V. As was the case with purified NR samples in Chapter 3 of these Results no protein-staining bands were detected making it impossible to assess the purity of the sample.

However, only one band was found to stain for NADH-NBT reductase activity with an R_f of 0.43 ± 0.02 (8 gels).

Characterisation of the 3.1S NADH-CR Species

Molecular Weight Determination

Small (1980) identified two major small NADH-CR species in crude barley leaf extracts by both sucrose density gradient centrifugation and gel filtration analysis. The smaller of the two species observed using both techniques was assumed to represent a single entity and a similar assumption was made regarding the larger of the two species. Molecular weights of 40 000 and 60 000 daltons were calculated for the two species from their sedimentation coefficients of 3.1 and 3.8S and Stokes radii of 3.1 and 3.4 nm respectively.

Small (1980) identified the major NADH-CR species in 168 h old barley leaf extracts by subjecting an aliquot of the peak NADH-CR containing fraction from Biogel A 1.5 m chromatography to sucrose density gradient analysis. The predominant NADH-CR species was found to have a sedimentation coefficient of 3.1S. Since the 3.1S species in crude extracts had previously been calculated to have a molecular weight of 40 000 it was assumed that the predominant NADH-CR species on Biogel A 1.5 m chromatography also had a molecular weight of 40 000. In an attempt to confirm this directly on the same sample, purified NR related NADH-CR species was subjected to sucrose density gradient centrifugation and gel filtration analysis (Figs.29 and 30 respectively).

Both techniques revealed apparently homogeneous peaks of NADH-CR activity. This NADH-CR species was found to have a sedimentation coefficient of 3.0S and a stokes radius of 3.2 nm giving a calculated molecular weight of 39 613 daltons thus confirming the assumptions made by Small (1980).

A molecular weight of 38 000 has subsequently been calculated directly for this NADH-CR species in this laboratory by SDS gel electrophoresis (J.McA. Campbell *et al*, 1983) however this NADH-CR species will be referred to hereafter as the 40 000 MW NADH-CR species.

Fig.29

Sucrose Density Gradient Centrifugation Analysis of a Sample of Purified 3.1S NADH-CR Species

This figure shows the distribution of NADH-CR activity (●) and NADH-NBT reductase activity (o) after a 0.4 ml sample of purified 3.1S. NADH-CR species was analysed on 2-18% sucrose gradients.

C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.

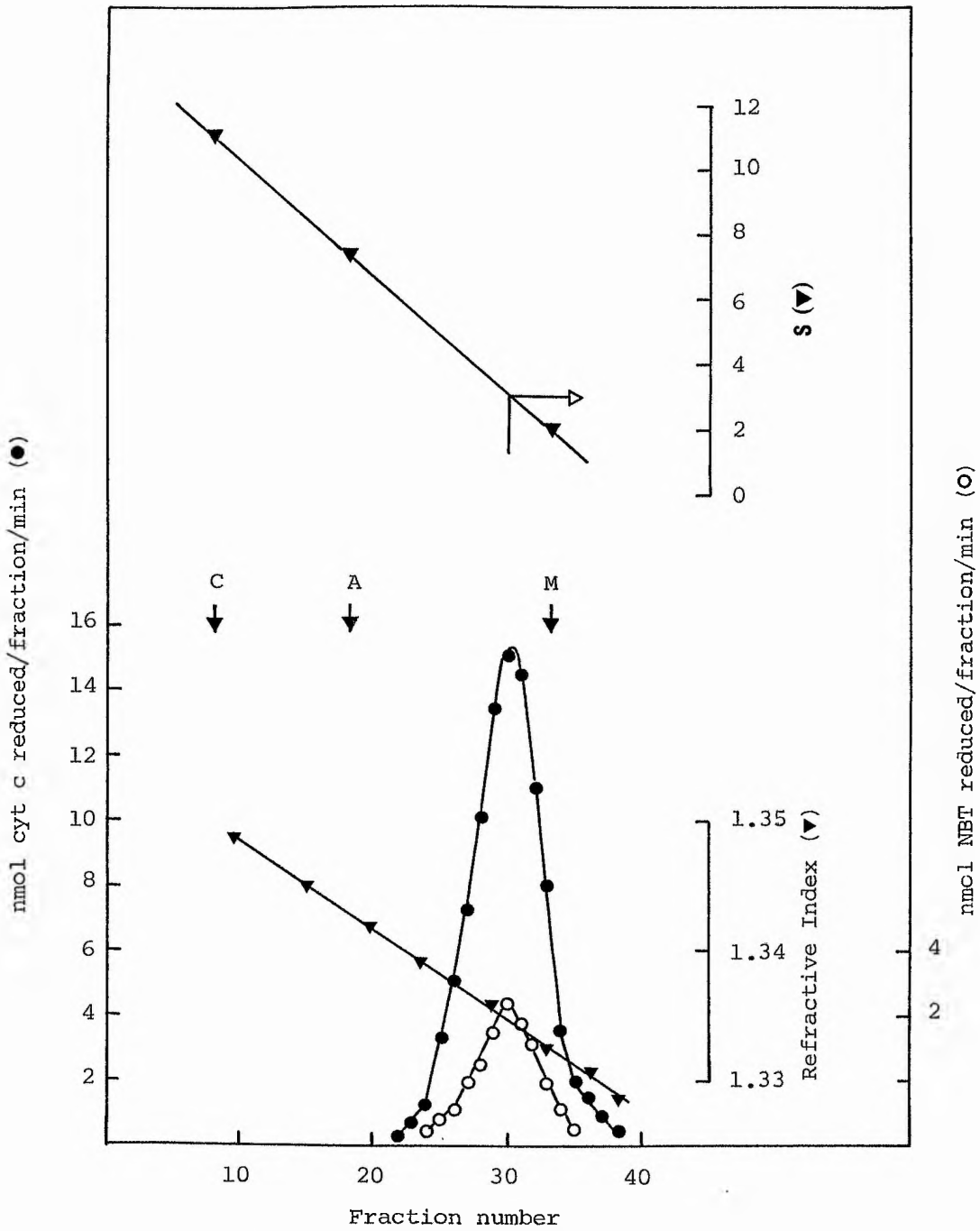
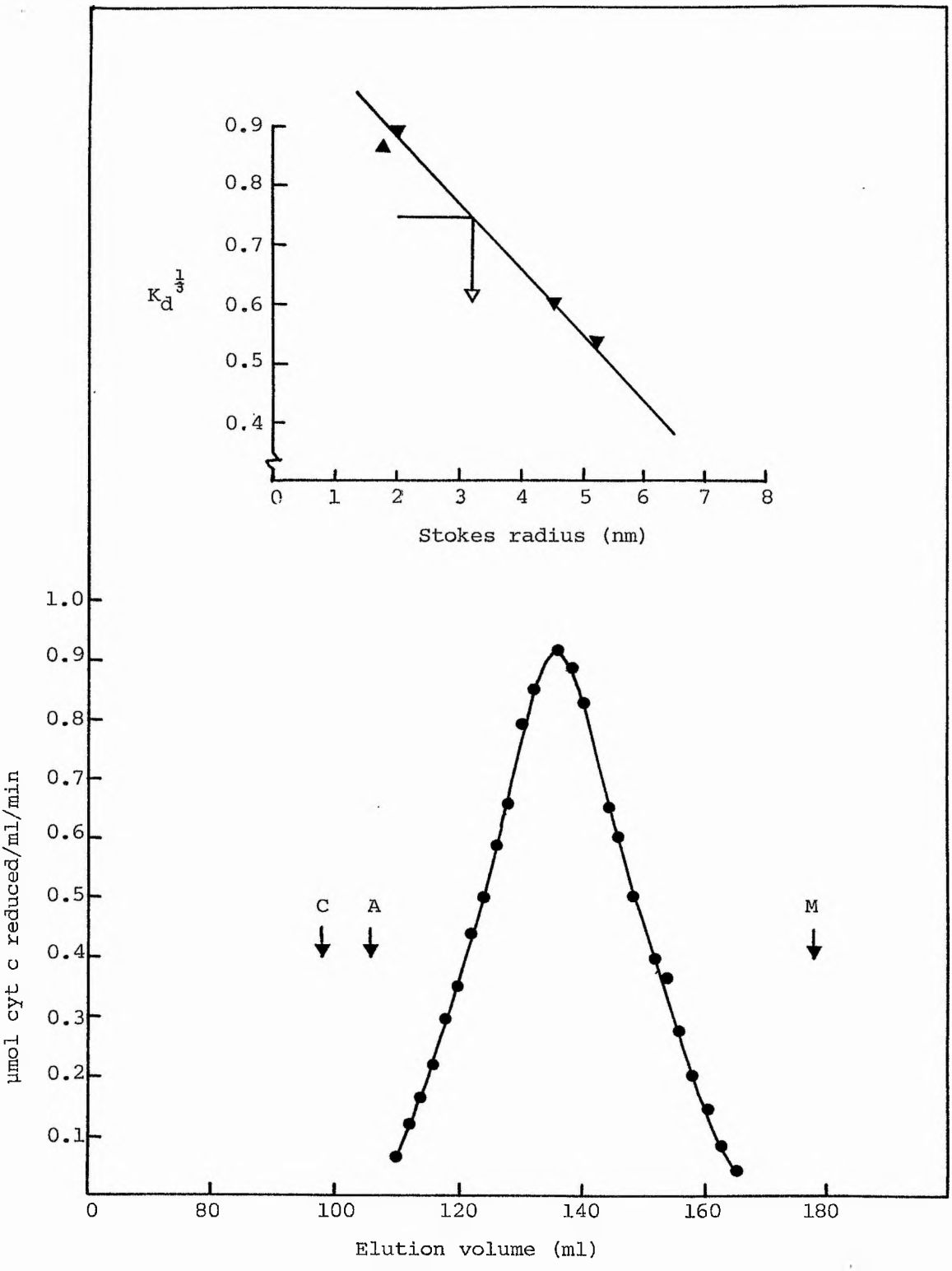

nmol cyt c reduced/fraction/min (●)
nmol NBT reduced/fraction/min (○)
S (▶)
Refractive Index (▶)
C
A
M
Fraction number
16
14
12
10
8
6
4
2
10
20
30
40
12
10
8
6
4
2
0
1.35
1.34
1.33
4
2

Fig.30

Sephadex G200 Gel Filtration of a Sample of Purified 3.1S NADH-CR species

This figure shows the distribution of NADH-CR activity following Sephadex G200 gel filtration of a sample of purified 3.1S NADH-CR species.

C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin.


0.9
0.8
0.7
0.6
0.5
0.4
$K_d^{\frac{1}{3}}$
0 1 2 3 4 5 6 7 8
Stokes radius (nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
µmol cyt c reduced/ml/min
C
A
M
0 80 100 120 140 160 180
Elution volume (ml)

NADH-Nitroblue Tetrazolium (NBT) Reductase Activity of the 40 000 MW NADH-CR Species

Hewitt (1975) and others have made assumptions that NBT was capable of acting as a substrate for the dehydrogenase activity of higher plant NR. This was confirmed by Small (1980) using crude and purified preparations of barley NR. The purified NR preparations used by Small were contaminated with 40 000 MW NADH-CR species and he found that NBT was 8-fold more effective as a substrate for this NADH-CR species than as a substrate for the dehydrogenase activity of NR.

The distribution of NADH-NBT reductase activity following sucrose density gradient centrifugation of purified 40 000 MW NADH-CR species is presented in Fig.29. The peak of NADH-NBT reductase activity corresponds to the peak of CR at 3.0S but as the NBT reductase peak was smaller than the NADH-CR peak it would appear that cytochrome *c* is a much better substrate for the NADH-CR dehydrogenase activity than NBT.

It follows that the band of dehydrogenase activity observed on non-denaturing gel electrophoresis using NBT as substrate as described in the previous section, is likely to be due to the activity of the 40 000 MW NADH-CR species.

Assessment of Haem Presence in the 40 000 MW NADH-CR Species Preparation

There is dispute as to whether cyt b_{557} is involved in NADH-CR activity (Hewitt, 1975; Notton *et al*, 1977; Maldonado *et al*, 1978; Fido *et al*, 1979). If haem were absent from the purified 40 000 MW NADH-CR preparation then it could not be essential for CR activity but this would not rule out the possibility that cytochrome may be involved when CR activity is expressed by the intact NR complex.

50µl samples of PEG 6 000-concentrated, Blue Dextran Sepharose purified 40 000 MW NADH-CR species and NR (containing approximately 5µg protein) were subjected to non-denaturing gel electrophoresis in 5% acrylamide gels as previously described. Gels were stained for haem using the method of Thomas *et al* (1976). I was unable to stain either the 40 000 MW NADH-CR species or NR for haem which may have been due to the 3,3',5,5' tetramethyl benzidine (TMBZ) used which was difficult to keep in solution. Alternatively insufficient protein may have been loaded onto the gel for staining of haem to occur. In some of the 40 000 MW NADH-CR preparations however, heavy haem staining was observed in the R_f0-$R_f0.3$ region of the gel which did not correspond to NADH-NBT reductase activity and appeared to represent gross haem contamination of the sample or denatured haem-containing protein unable to enter the gel.

Haem has subsequently been shown to be absent from similarly prepared preparations of this 40 000 MW NADH-CR species in this laboratory by J.McA. Campbell *et al* (1983). The NADH-CR species could not be stained for haem in non-denaturing gels under conditions which stained both purified barley NR and equine heart cytochrome *c*.

CHAPTER 5

TRYPSIN TREATMENT OF PURIFIED BARLEY NITRATE REDUCTASE

The NR Substrate

NR purified according to the procedure outlined in Methods, Section II was used as the substrate for tryptic cleavage experiments. The peak NR-containing fractions from Blue Dextran Sepharose chromatography were pooled, placed on ice and used immediately. Although the extent of purity of this NR preparation could not be assessed due to difficulties with protein staining on polyacrylamide gels, it was thought likely to be inhomogeneous with respect to NR. However, this purified preparation of NR was found, on most occasions, to contain only trace amount of CR species sedimenting in the 3-4S region of sucrose gradients (Fig.32a). This is difficult to reconcile with the fact that NR was found to be highly unstable in this purified preparation even on ice, losing up to 40% of its initial activity during a 10 min incubation. Presumably this inactivation was not due to conversion of NR to smaller CR species, as observed in crude extracts (Chapter 2) but rather was a reflection of instability of NR in a low protein environment (Yamaya *et al*, 1980b), a situation which is not encountered in crude extracts. It is therefore clear that different factors may contribute to the instability of NR in purified and crude enzyme preparations.

Conditions for Tryptic Cleavage of NR

Purified NR was found to be extremely sensitive to inactivation by trypsin. In order to minimise

instability of NR in the control treatment, limited proteolysis was carried out on ice using a trypsin concentration which resulted in approximately 90% inactivation of NR activity within 9 min of incubation. The trypsin concentration varied with batches of NR substrate but with a typical NR preparation containing approximately 0.025 mg/ml protein, a final trypsin concentration of 0.1µg/ml in the incubation was employed resulting in a ratio of total protein in the preparation to trypsin concentration of 200:1.

Trypsin inhibitor (Egg white type II - O) was found to be effective in terminating inactivation of NR by trypsin under these conditions (data not shown). A final concentration of trypsin inhibitor which exceeded the manufacturers recommendations was however used as a precautionary measure (final concentration 5µg/ml).

Effect of Trypsin on the Partial Activities of NR

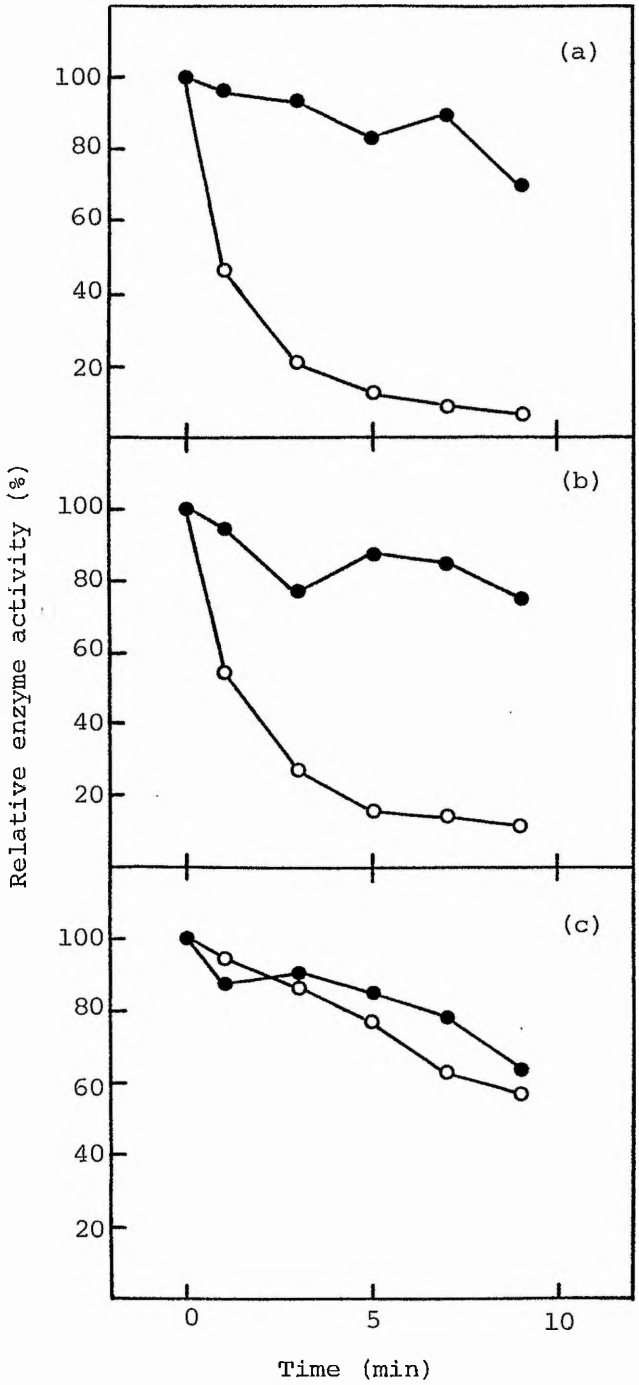
The time courses of inactivation of the NADH-NR, MVH-NR and NADH-CR activities of NR were studied as a means of determining which of the NR activities was most sensitive to trypsin and therefore the probable site of attack by the proteinase.

From the results presented in Fig.31 it appears that the NADH-CR activity is relatively resistant to inactivation by trypsin while NADH-NR and MVH-NR activities were rapidly abolished. There was an indication that

Fig.31

Effect of Trypsin on the Partial Activities of NR

0.9 ml of purified NR preparation (1.06µmol nitrite produced/ml/h) were incubated, on ice, with 0.1 ml of Buffer III (•) or 0.1 ml of Buffer III containing 1µg/ml trypsin (o). The final concentration of trypsin was 0.1µg/ml. 0.1 ml aliquots of the mixtures were removed into 0.1 ml of trypsin inhibitor in Buffer III (10µg/ml) at the time intervals indicated before assaying for residual NADH-NR (a), MVH-NR (b) and NADH-CR (c) activities.


(a)
(b)
(c)
100
80
60
40
20
0
5
10
Relative enzyme activity (%)
Time (min)

MVH-NR activity may be slightly more resistant to tryptic cleavage than NADH-NR, but due to the very rapid inactivation this was difficult to assess. Since the NR substrate is likely to be free from constitutive CR species one must assume that the NADH-CR activity which is stable to tryptic cleavage is none-the-less derived from NR.

Sucrose Density Gradient Analysis of the Products of Tryptic Cleavage

Purified NR preparations before and after exposure to trypsin were analysed on sucrose gradients (Fig.32).

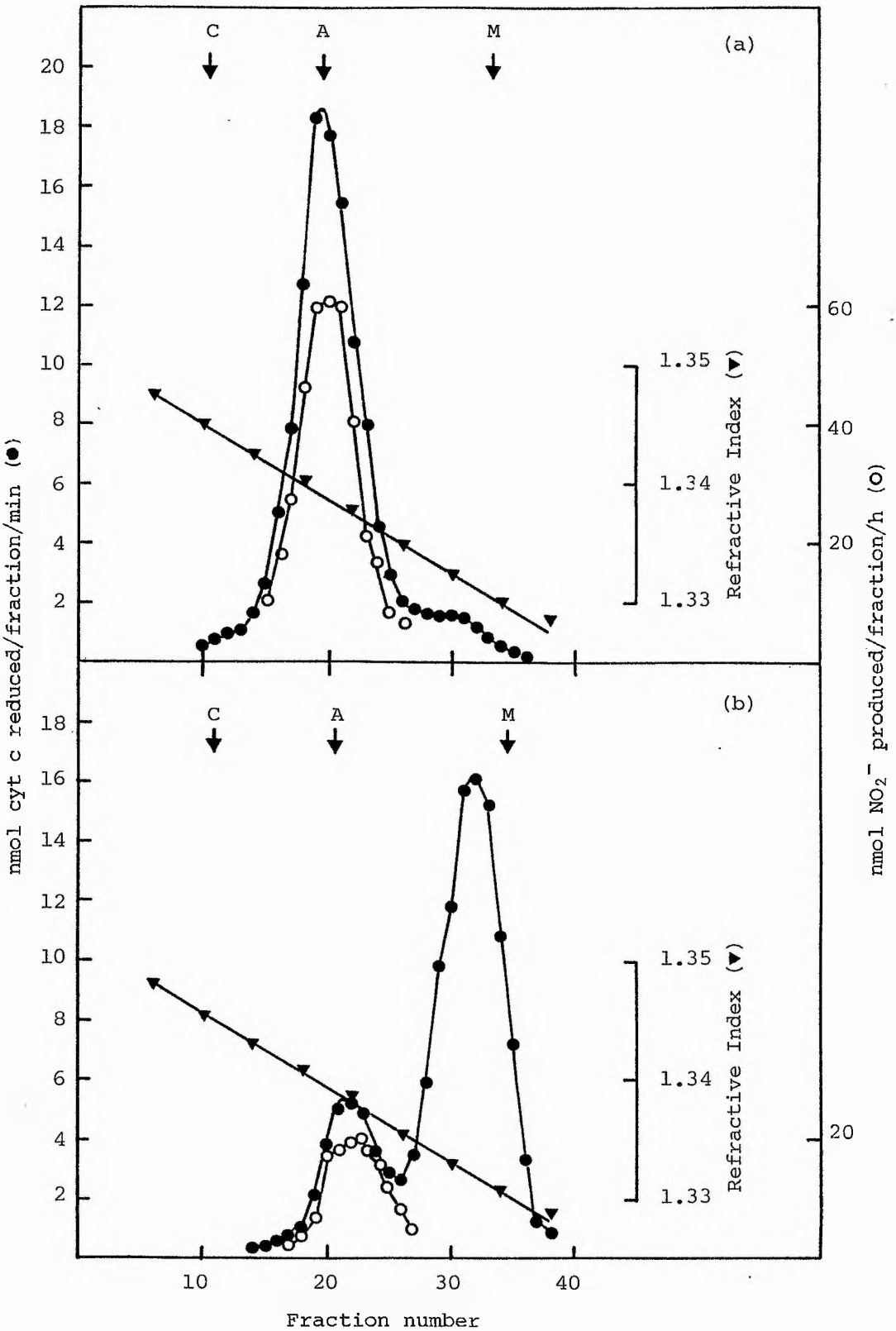
The total NADH-CR activities recovered from both gradients were very similar yet the distribution of NADH-CR species was strikingly different. As discussed previously, the major NADH-CR species in this purified NR preparation sediments at 7.7S with a very small amount of NADH-CR species sedimenting in the 3-4S region of the gradient (Fig.32a). However, tryptic cleavage appears to convert 7.7S NADH-CR species (intact NR), very rapidly into large amounts of the 3.1S NADH-CR species and small amounts of the 6.8S and 3.8S NADH-CR species with little apparent loss of total NADH-CR activity (Fig.32b). This statement does, of course, assume that the diaphorase function of the NADH-CR species is equally active when it is part of the intact enzyme as when it has been cleaved from the complex.

Fig.32

Sucrose Density Gradient Centrifugation Analysis of Purified NR Before and After Exposure to Trypsin

0.9 ml of purified NR preparation (7.49µmol nitrite produced/ml/h) were incubated, on ice, with 0.1 ml of Buffer III or 0.1 ml of Buffer III containing 5µg/ml trypsin. After 10 min, 1 ml of trypsin inhibitor (1 mg/ml Buffer III) was added to each mixture to stop the inactivation.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after sucrose density gradient centrifugation analysis of purified NR (a) and purified NR exposed to tryptic cleavage (b). C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


C
A
M
(a)
(b)
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)
Refractive Index (▼)
1.35
1.34
1.33
Fraction number
10
20
30
40

It does appear that trypsin can generate the same NADH-CR species which have been observed on analysis of cell free extracts of primary leaves of barley but there is no evidence for any intermediate NADH-CR species sedimenting between 7.7S/6.8S and 3.8S/3.1S such as the 5.6S species which has also been observed (Small, 1980).

From these data one might submit that limited proteolysis may be a component of the proposed *in vitro* inactivation mechanism observed in cell-free extracts. There is, however, a major difference between the NADH-CR activity profiles after sucrose density gradient analysis of purified NR treated with trypsin and cell free extract of older leaf tissue. In crude extracts one never sees the large amounts of NADH-CR species sedimenting in the 3-4S region of the gradient that one sees on trypsin treatment. It is possible that the subsequent action of proteinases on the degradation products of NR in cell-free extracts results in there being no build up of small NADH-CR species.

The Effect of BSA on the Limited Proteolysis of NR by Trypsin

It appears that the tryptic products of NR, which have NADH-CR activity, are the same as those generated in cell-free extracts. Inclusion of BSA in the extraction buffer prevented the conversion of NR into smaller NADH-CR species in cell-free extracts. It has been proposed that

BSA does this by acting as an alternative substrate for proteinases. If this is the case, one might expect 3% BSA to protect purified NR from inactivation by trypsin, and this proposal was investigated (Fig.33a).

3% BSA increased the stability of NR activity over that in the control treatment, which was to be expected since purified NR is more stable in a higher protein environment (Yamaya *et al*, 1980b). However, 3% BSA does appear to protect NR from inactivation by trypsin at 0-4°C. It is not clear from this data whether it does so by acting as an alternative substrate for trypsin or by making NR more stable and thus less susceptible to inactivation by trypsin which is, of course, also true for the situation in crude extracts.

When the experiment described above was repeated using 0.3% (w/v) BSA instead of 3% (w/v) BSA (Fig.33b), protection from inactivation of NR by trypsin was dramatically reduced while 0.3% BSA stabilised NR in the control treatment to the same extent as that of 3% BSA. The results described here agree with those of Yamaya *et al* (1980b) who showed that 0.2% (w/v) BSA had no influence on the inactivating activity of trypsin on *Chlorella* NR while stabilising NR in the absence of trypsin. It would appear that the mechanism whereby BSA stabilises NR against tryptic cleavage is dependent on the non-plant protein being present at very high concentration whereas even low concentrations of BSA are capable of stabilising

Fig.33

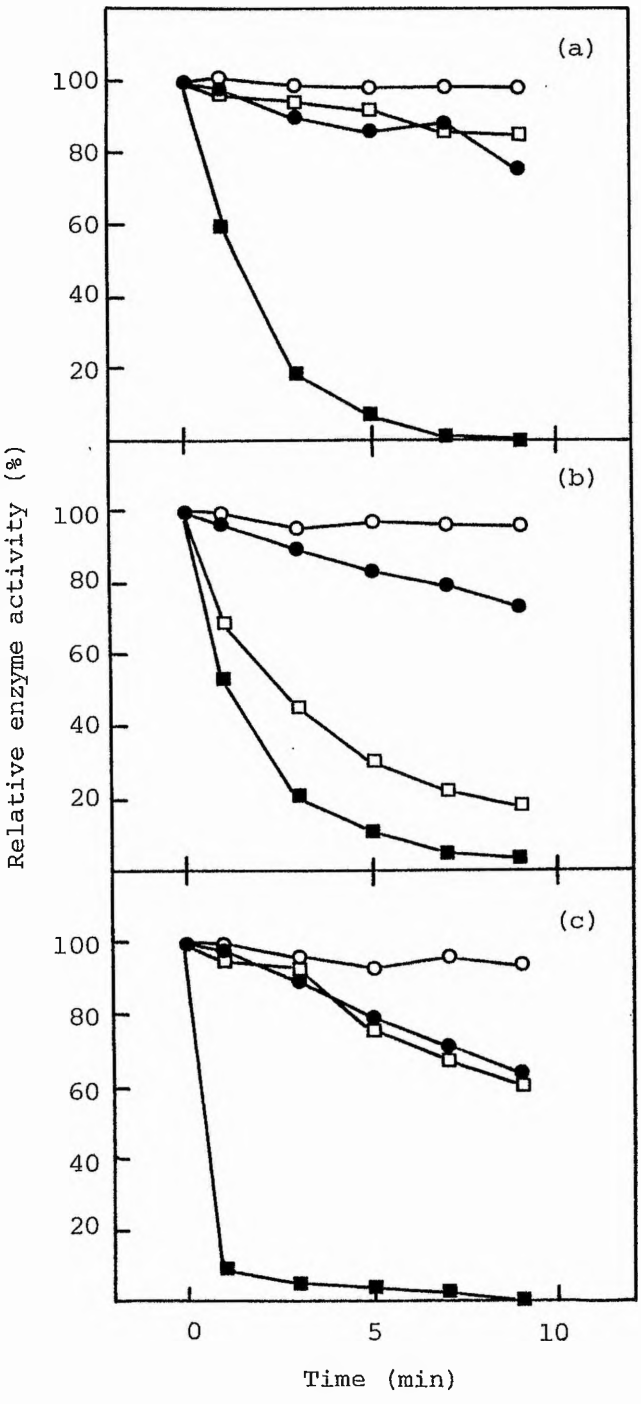
Effect of 3% and 0.3% (w/v) BSA on Tryptic Cleavage of NR at 0-4 °C and 3% (w/v) BSA at 25°C

(a) 0.6 ml of NR substrate (1.06µmol nitrite produced/ml/h) were incubated at 0-4°C with: 0.4 ml of Buffer III (●); 0.1 ml of Buffer III and 0.3 ml of Buffer III containing 10% (w/v) BSA (○); 0.1 ml of Buffer III containing 1µg/ml trypsin and 0.3 ml of Buffer III (■); 0.1 ml of Buffer III containing 1µg/ml trypsin and 0.3 ml of Buffer III containing 10% (w/v) BSA (□).

0.1 ml aliquots of the mixtures were removed into 0.1 ml of 10µg/ml trypsin inhibitor in Buffer III at time intervals before assaying for NADH-NR activity.

(b) Conditions as for (a) except Buffer III containing 1% BSA was used.

(c) Conditions as for (a) except incubation was at 25°C.


(a)
(b)
(c)
100
80
60
40
20
Relative enzyme activity (%)
0
5
10
Time (min)

NR observed in the absence of trypsin, in a low protein environment.

At 25 °C (Fig.33c), NR in the control treatment was less stable than at 0-4 °C. NR appeared more susceptible to inactivation by trypsin at this higher temperature or rather trypsin was more active at this temperature. The protective effect of 3% BSA on NR inactivation by trypsin was not significantly reduced at 25°C. A reduction in stabilising ability of BSA on NR at higher temperature has previously been noted in cell-free extracts (Chapter II, Fig.18) but due to the mode of the process this did not become apparent until after 15 min incubation at 25°C so it is therefore unlikely to be observed over the 9 min time course of trypic cleavage described here.

Effect of BSA on Tryptic Cleavage of NR Analysed by Sucrose Density Gradient Centrifugation

Tryptic cleavage in the presence and absence of BSA was performed essentially as described previously but terminated at 4 min and the resulting samples analysed with a no trypsin, no BSA control on sucrose gradients (Fig.34).

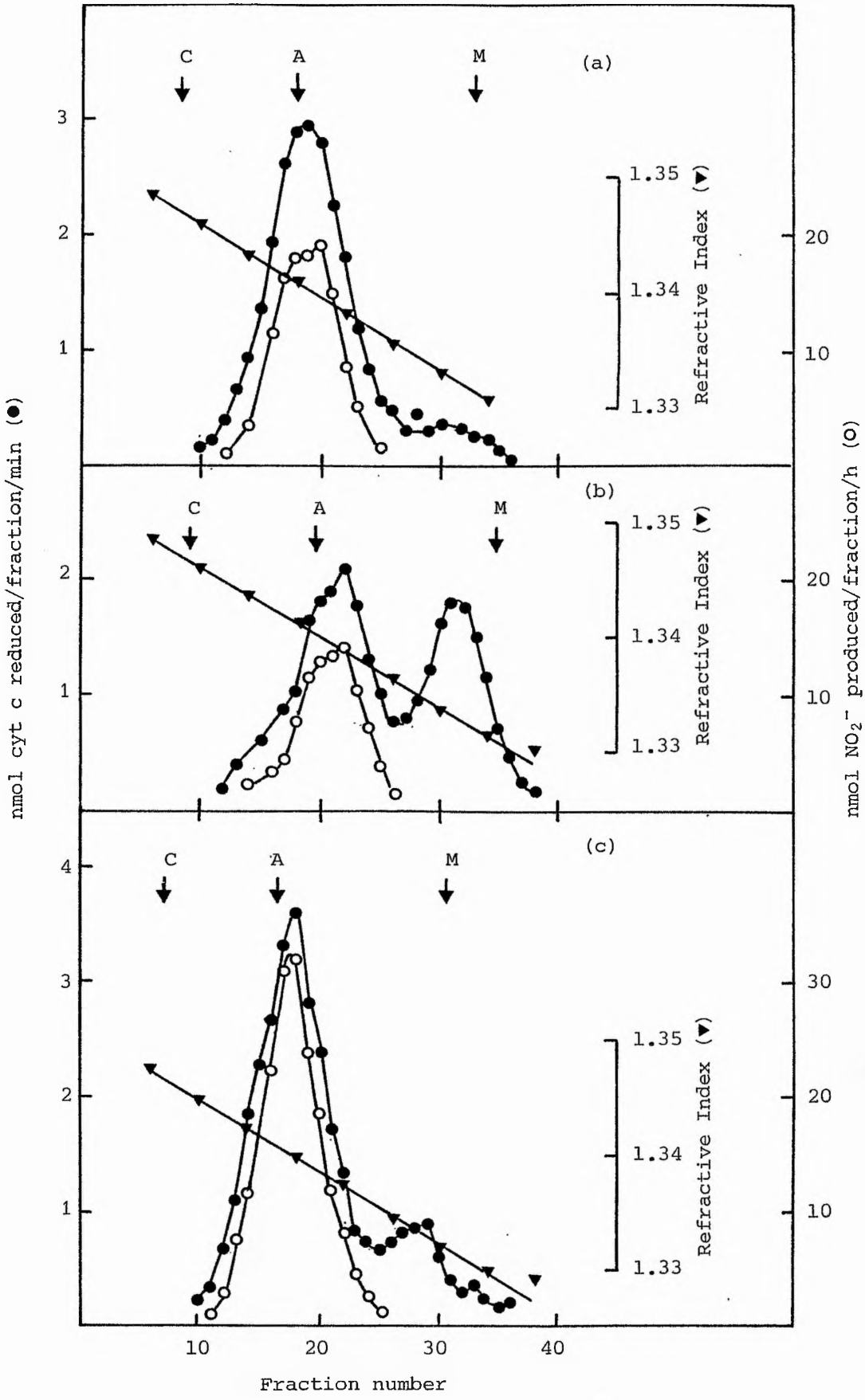
The major CR species in the control treatment (Fig.34a) sedimented at 7.7S with little breakdown into smaller NADH-CR species. The trypsin only treatment (Fig.34b) showed an intermediate NADH-CR activity profile to that shown previously after 10 min incubation with trypsin

Fig.34

Sucrose Density Gradient Analysis of Untreated Purified NR and NR Exposed to Trypsin in the Presence and Absence of 3% BSA

0.6 ml of the NR substrate (2.57µmol nitrite produced/ml/h) were incubated on ice with either 0.4 ml of Buffer III, 0.4 ml of Buffer III containing 0.25µg/ml trypsin or 0.4 ml of Buffer III containing 0.25µg/ml trypsin and 3% (w/v) BSA. After 4 min incubation on ice, 1 ml of 10µg/ml trypsin inhibitor in Buffer III was added to each mixture.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after 0.4 ml aliquots of untreated NR (a), NR exposed to trypsin (b), and NR exposed to trypsin in the presence of 3% BSA (c), were analysed on 2-18% sucrose gradients. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


C
A
M
(a)
(b)
(c)
1.35
1.34
1.33
Refractive Index (▼)
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)
Fraction number
10
20
30
40

which is presumably a result of the reduced time in contact with trypsin and as such resembled the NADH-CR activity profile from 5 day old primary leaf extract (Fig.34b).

The major NADH-CR species in the trypsin plus BSA treatment (Fig.34c) is the 7.7S species of which there was more than in the control treatment. This is likely to be a reflection of the stabilising effect of BSA on NR activity in the control treatment observed previously (Fig.34a). In addition, more NADH-CR species sediment in the 3-4S region of the gradient than in the control treatment, confirming the observation noted previously that BSA does not completely prevent inactivation of NR by trypsin.

CHAPTER 6

TREATMENT OF PURIFIED BARLEY NITRATE REDUCTASE WITH MAIZE ROOT NITRATE REDUCTASE INACTIVATOR I AND A BARLEY LEAF NITRATE REDUCTASE INACTIVATOR

Investigation into the Existence of Specific Inactivation Mechanism(s) of Barley Leaf NR

Indirect evidence for the existence of proteinases active towards NR comes from the observation that NR activity is unstable in crude extract and that various proteinase inhibitors retard, and BSA prevents, the loss of activity. In addition, trypsin cleaves purified NR into the same NADH-CR species observed in crude extracts. To what extent these *in vitro* phenomena relate to possible *in vivo* regulation of NR levels by proteolytic degradation is not known. There is however abundant evidence to show that plants possess proteinases potentially capable of degrading NR *in vivo*.

Purification and Characterisation of Maize Root NR Inactivators (according to Smith, 1983)

Maize root inactivators of NR have been purified in this laboratory by Smith (1983) essentially according to the method of Wallace (1974). Her findings regarding the purification and characterisation of two inactivators are summarised below.

The inactivators were purified from the mature root region of 5 day old seedlings by ammonium sulphate fractionation (40-70%), pH 4.0 precipitation and ion exchange chromatography on CM52 cellulose. NR inactivator I was eluted from the CM-cellulose column by 10mM acetate pH 5 containing 50mM NaCl and NR inactivator II was separated

by sequential elution with 10mM potassium phosphate pH 8.0.

NR inactivator I and the NR inactivating enzyme described by Wallace behaved similarly during CM-cellulose chromatography, were both able to degrade azocasein and were inhibited by PMSF and casein suggesting that the two factors were analagous. BSA and leupeptin (an inhibitor of those trypsin-like serine and cysteine proteinases which cleave peptide bonds at the carboxyl side of arginine and lysine residues) were unable to inhibit NR inactivator I. NR inactivator II differed in its response to inhibitors since it was inhibited by EDTA and 1,10-phenanthroline and only very slightly by PMSF but not by casein, BSA or leupeptin. In addition, it was unable to degrade azocasein.

Both inactivators inactivated NADH-NR activity most rapidly closely followed by NADH-CR activity. NR inactivator I inactivated MVH-NR activity at a slower rate whereas this activity was completely unaffected by NR inactivator II.

Effect of Maize Root NR Inactivator I on Purified Barley NR Analysed by Sucrose Density Gradient Centrifugation

A sample of maize NR inactivator I (purified up to the CM-52 cellulose stage) was kindly provided by Miss S.C. Smith in order to analyse, by sucrose density gradient centrifugation, the products of inactivation of

purified barley NR (Fig.35). The NR substrate used was as previously described for tryptic cleavage analysis namely the pooled peak NR-containing fractions from Blue Dextran Sepharose chromatography.

Treatment of NR with the maize root proteinase resulted in major loss of the 7.7S NADH-CR species which represents the intact NR complex. However in contrast to trypsin treatment of purified NR, loss of 7.7S NADH-CR activity was not accompanied by an increase in smaller NADH-CR species. Such results are consistent with the findings of Wallace (1975b) that the maize root proteinase preferentially attacks the part of the NR complex which carried NADH-CR activity. Since there were only trace amounts of the small NADH-CR species present in the control treatment, the inactivating enzyme's effect on the 3.1S species could not be assessed.

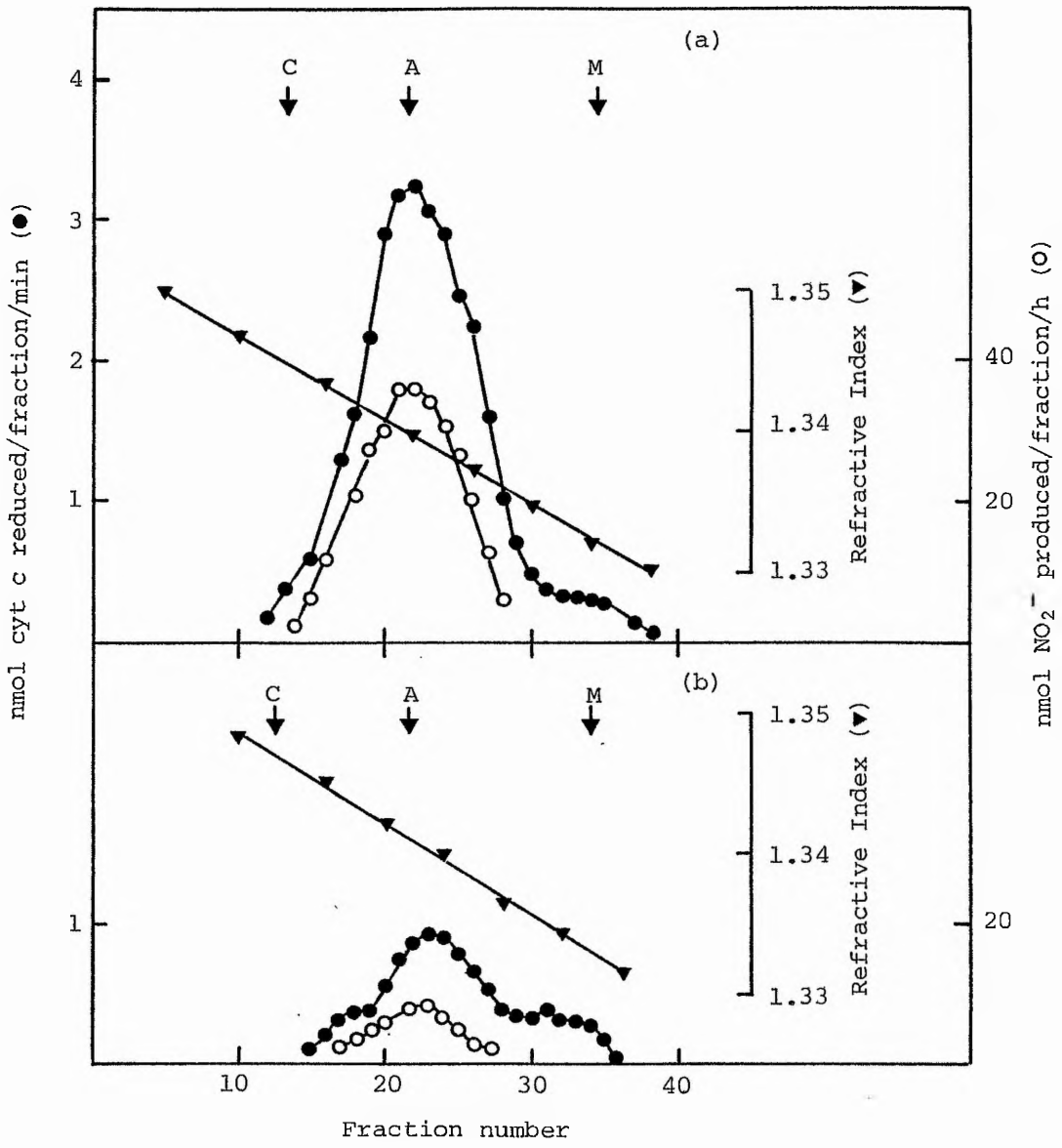
The loss of MVH-NR activity on incubation with the maize root proteinase is known to occur at a slower rate than NADH-NR and NADH-CR (Smith, 1983) but unfortunately this activity was not assayed on the gradients on this occasion. However Smith (1983) has shown that a peak of MVH-NR activity was retained on sucrose gradients after all NADH-NR activity was abolished using purified maize NR as substrate.

Fig.35

Sucrose Density Gradient Analysis of Purified Barley NR Before and After Exposure to Maize Root NR Inactivator I

0.8 ml of purified barley NR (2.51µmol nitrite produced/ml/h) were mixed with 0.1 ml of purified maize NR inactivator I (14µg protein) dissolved in Buffer III. 0.1 ml of Buffer III containing 5mM PMSF was added either immediately or after 10 min incubation on ice to inhibit NR inactivation.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after analysis of 0.4 ml aliquots of barley NR before (a) and after treatment (b) on 2-18% sucrose gradients. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


(a)
C
A
M
4
3
2
1
1.35
1.34
1.33
Refractive Index (▼)
40
20
(b)
C
A
M
1
1.35
1.34
1.33
Refractive Index (▼)
20
10
20
30
40
Fraction number
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)

Isolation and Characterisation of Barley NR Inactivators (according to Smith (1983)

Smith (1983) succeeded in isolating NR inactivating activity from 5 day old barley roots by the method described for maize NR inactivators. The equivalent of the NR inactivator I was, however, completely absent and only one peak of NR inactivating activity was detected and this was eluted by 10mM potassium phosphate pH 8.0.

Smith also obtained the same pattern of NR inactivating activities from 7 day old barley shoots treated similarly. The barley leaf NR inactivator was found to be inhibited by EDTA, 1,10-phenanthroline and to a lesser, but significant, extent by leupeptin. Its activity was not affected by BSA, casein or PMSF and resembled maize root inactivating factor II.

Effect of Barley Leaf NR Inactivator on Purified Barley NR Analysed by Sucrose Density Gradient Centrifugation

A sample of the barley leaf NR inactivating factor was kindly provided by Miss S.C. Smith in order to analyse, by sucrose density gradient centrifugation, the inactivation products of purified barley NR (Fig.36).

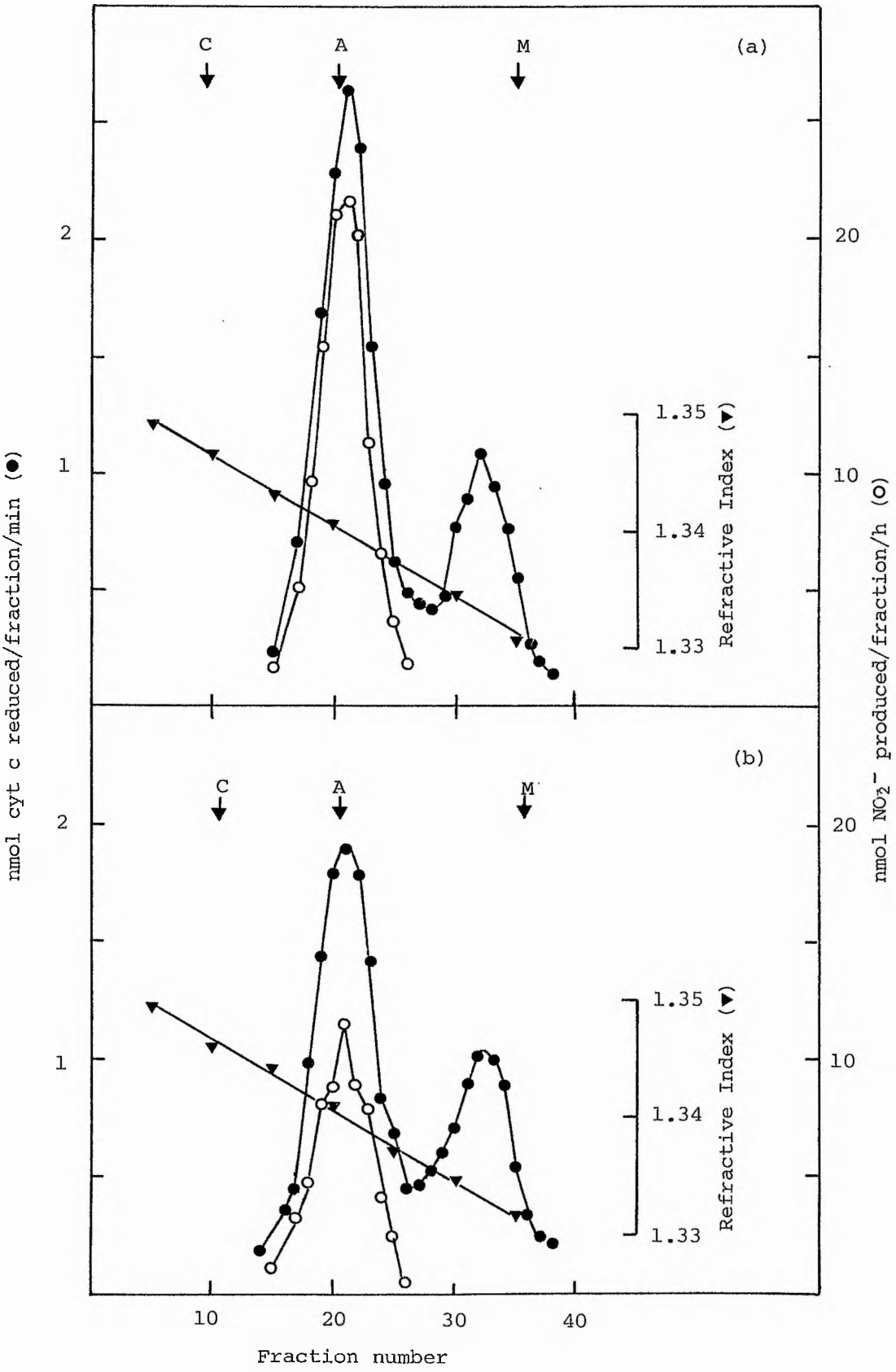
The most striking feature of the control treatment (Fig.36a) is the amount of NADH-CR activity sedimenting in the 3-4S region of the gradient as previous analyses of the NR substrate showed only trace amounts. This could be due to insufficient 1,10-phenanthroline in the mixtures

Fig.36

Sucrose Density Gradient Analysis of Purified Barley NR Before and After Exposure to the Barley Leaf NR Inactivator

1 ml of purified barley NR (0.95µmol nitrite produced/ml/h) was mixed with 280µl of barley leaf NR inactivator (224µg protein) dissolved in Buffer III. 20µl of 200mM 1,10-phenanthroline was either added immediately or after incubation for 60 min at 25°C to inhibit NR inactivation.

This figure shows the distribution of NADH-NR (o) and NADH-CR (•) activities after 0.4 ml aliquots of barley NR before (a) and after (b) inactivation were analysed on 2-18% sucrose gradients. C, A and M denote the respective positions of the reference proteins catalase, alcohol dehydrogenase and myoglobin after centrifugation.


C
A
M
(a)
(b)
2
1
20
10
1.35
1.34
1.33
Refractive Index (▼)
nmol cyt c reduced/fraction/min (●)
nmol NO_2^- produced/fraction/h (○)
10
20
30
40
Fraction number

or alternatively it is possible that the inactivator preparation was not homogeneous since leupeptin was also shown to affect activity. As 1,10-phenanthroline was the only proteinase inhibitor included in the incubations it is possible that other inactivators were able to act upon the NR substrate.

After exposure to barley NR inactivator for 60 min (Fig.36b) the NADH-CR activity of the 7.7S NR complex was reduced while the activity of the 3.1S NADH-CR species remained unchanged. No MVH-NR activities were measured but it appears that the NR inactivator sensitive to 1,10-phenanthroline in the preparation does not cause net degradation of NR to smaller, stable NADH-CR species since the 3.1S NADH-CR species did not accumulate.

No time course data was available for the effect of barley NR inactivator on purified NR but if one assumes that the species is analagous to the NR inactivator II from maize roots (by comparison of properties as described above) then one might expect no accumulation of small NADH-CR species since the inactivator was most active towards the diaphorase function of the enzyme. In addition, the barley inactivator might not be expected to degrade NR at all since MVH-NR activity was totally resistant to maize root inactivator II.

It appears from the data presented here that the proteolytic mechanisms of the NR inactivators investigated differ from that of trypsin since, unlike trypsin, they are unable to generate the same small CR species as observed in crude extracts. Maize NR inactivator I

(and II) and barley NR inactivator-type enzymes may, however, contribute to the instability of NR in crude extracts since PMSF and 1,10-phenanthroline were shown to retard the loss of NR activity to some extent (Results, Chapter II).

CHAPTER 7

STUDIES WITH ANTISERA RAISED AGAINST BARLEY NITRATE REDUCTASE AND THE 40 000 MW NADH-CYTOCHROME *c* REDUCTASE SPECIES

Use of Anti-NR Serum as a Tool for Investigation of NR

Assays for the complete and partial activities of NR have provided sensitive probes for investigating various aspects of NR structure and regulation. However, in certain circumstances, it is important to determine the extent to which NR protein is present irrespective of its catalytic activity, for example when it is necessary to distinguish between an irreversible degradation of NR and reversible inactivation. In addition, it would be of interest to detect breakdown products of NR which may not possess the potential for expressing any enzyme activity. Immunochemical techniques potentially provide specific and sensitive methods for both qualitative and quantitative analysis of NR.

Production of Antisera Against Purified NR and the 40 000 MW NADH-CR Species

NR was purified by the procedure outlined in Methods, Section VI. The peak NR-containing fractions from Blue Dextran Sepharose chromatography were pooled and concentrated (10-fold) against PEG 6 000 at 4°C. Antibodies against this purified NR were raised in a female, Dutch rabbit by subcutaneous injection of 100µg of the NR preparation emulsified in Freund's complete adjuvant. 25 days later an additional 20µg of NR preparation in an incomplete adjuvant mixture (see Methods Section VI for details) was injected. After 18 days and at various time intervals after the booster injection,

24 ml blood samples were collected from the ear vein of the rabbit, the serum separated and stored in aliquots at -70°C until used. When all samples had been collected, aliquots of each serum sample were thawed at room temperature and their antibody titre estimated by assessing their ability to inhibit NR in a purified enzyme preparation (see legend to Fig.37a for details of this procedure).

Antiserum against Blue Dextran Sepharose purified 40 000 MW NADH-CR species was raised using an identical procedure to that described above for NR. Antibody titre of the serum samples was estimated by assessing their ability to inhibit NADH-CR activity in a purified preparation of 40 000 MW NADH-CR species (see legend to Fig.37b for details of this procedure).

The µg quantities of purified NR and 40 000 MW NADH-CR preparations used for injections were sufficient to elicit an antibody response, especially after the booster injection. The titre of the anti-NR serum fell off rapidly over the subsequent 50 days while that of the anti-NADH-CR species serum remained relatively high (Fig.37).

Ouchterlony Double Diffusion Analysis of the Antisera

The precipitation reaction between anti-NR serum and purified NR and anti-40 000 MW NADH-CR species serum and purified 40 000 MW NADH-CR species was examined using the Ouchterlony gel diffusion system described in the Methods, Section VIII.

Fig.37

Titre of Serum Samples Obtained from Rabbits Immunised with NR and 40 000 MW NADH-CR Species

Antibodies against NR and 40 000 MW NADH-CR species were raised by the procedure outlined in the text. Serum prepared from blood samples collected during this process, which had been stored at -70°C, were thawed at room temperature and their antibody titre estimated.

(a) Estimation of titre of anti-NR sera

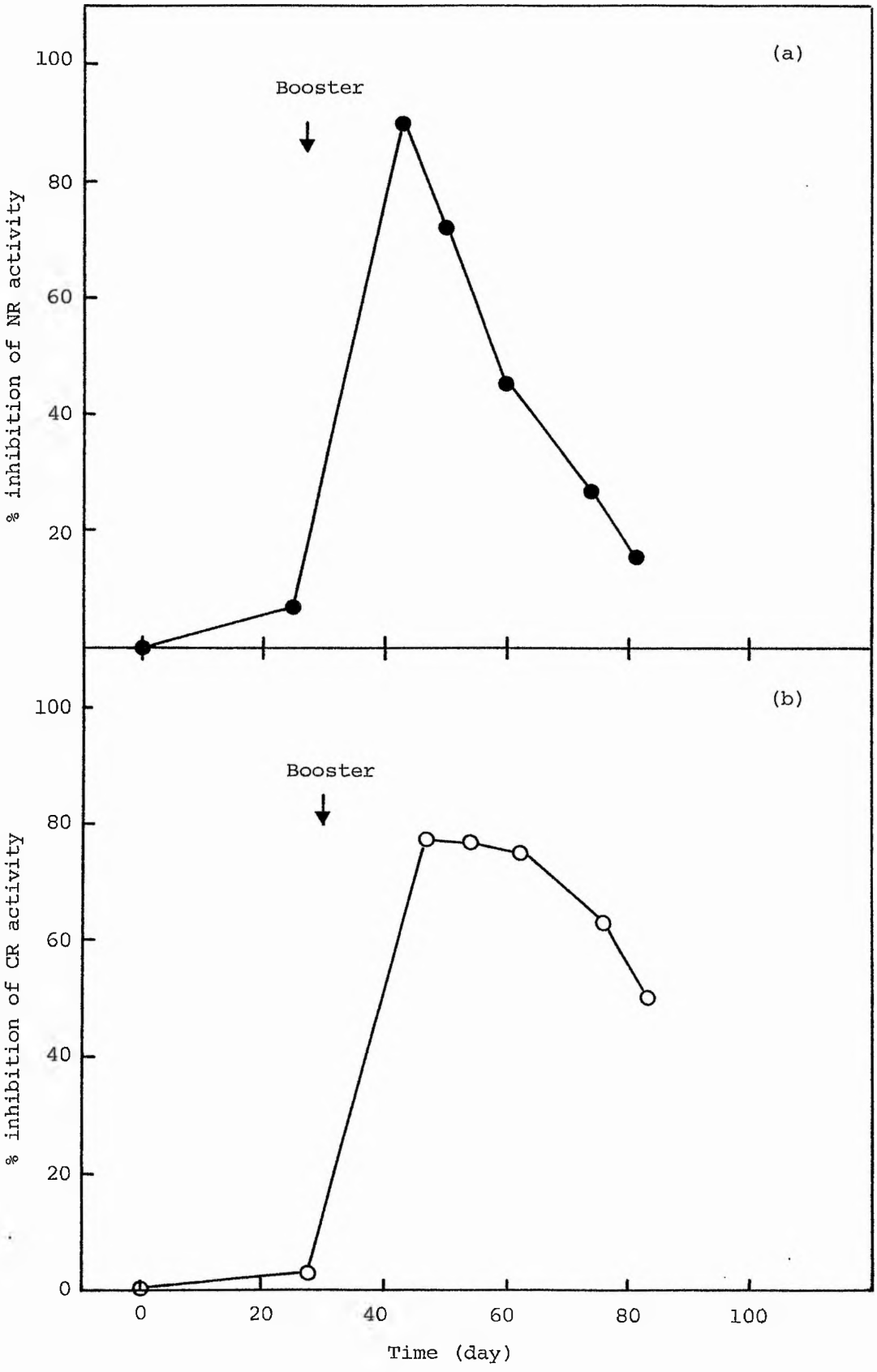
180µl of purified NR (0.04µmol nitrite produced/ml/h) were incubated with 20µl aliquots of each sample of serum and maintained at room temperature for 10 min before assaying for NR activity.

(b) Estimation of titre of anti-40 000 MW NADH-CR sera

180µl of purified 40 000 MW CR species (10.95 nmol cytochrome C reduced/ml/min) were incubated with 20µl of each sample of serum and maintained at room temperature for 10 min before assaying for CR activity.

The residual enzyme activity remaining in the mixtures expressed as a percentage of that in similarly treated controls which contained pre-immune serum was taken as an estimate of the titre of each serum sample. Percentage inhibition was then calculated by subtracting the % activity remaining from 100%.

This figure shows the percentage inhibition of NR activity by anti-NR sera (a) and % inhibition of NADH-CR activity by 40 000 MW NADH-CR species sera (b) collected on the days indicated.


(a)
Booster
100
80
60
40
20
% inhibition of NR activity
(b)
Booster
100
80
60
40
20
0
% inhibition of CR activity
0
20
40
60
80
100
Time (day)

No precipitin bands were observed when employing either decreasing concentrations of antigen (outer wells) with a constant volume of antiserum (centre well) or decreasing concentrations of antiserum (outer wells) with a constant volume of antigen (centre well). In retrospect, the implications of the non-linear antigen/antibody reaction were not fully appreciated at the time and it is likely that the optimum conditions for precipitation were not achieved.

Due to the limited time available it was decided to assume that the antisera were not monospecific and no attempt was made to further purify the antisera. The scope of possible investigations was therefore restricted.

Inhibition of Barley NR and Associated Activities by the Antiserum Directed Against NR

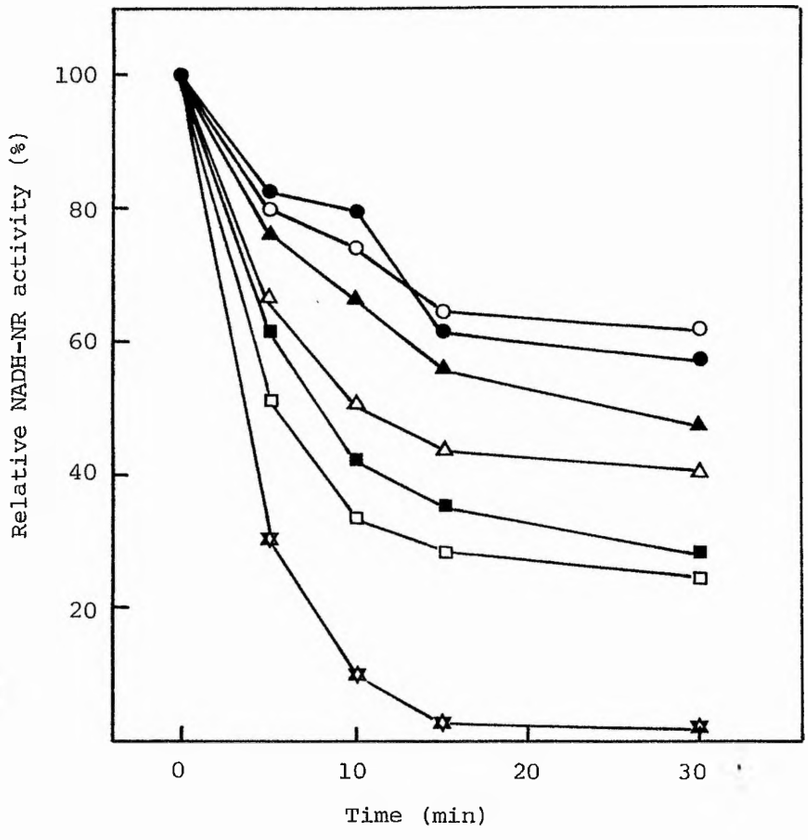
NR was purified according to the procedure outlined in the Methods, Section II. The peak NR-containing fractions from Blue Dextran Sepharose chromatography were pooled and placed on ice. Since this NR preparation has been shown to be highly unstable, even at 0-4°C (Chapter 5), it was used as soon as possible after preparation for inhibition studies and no attempt was made to standardise the activity of the preparation, which typically contained 1-5µmol nitrite produced/ml/h and 10-40µg/ml protein.

Inactivation of 100µl of purified NR by 0-10µl anti-NR serum was found to reach an end point after

Fig.38

Determination of a Suitable Incubation Time for Inhibition of NR by Anti-NR Serum Employing a Purified Preparation of NR

100µl of purified NR (1.57µmol nitrite produced/ml/h) were incubated with 0 (●), 1 (○), 2 (▲), 3 (△), 4 (■), 5 (□) and 10 (✡) µl anti-NR serum in a final volume of 200µl with Buffer III. The mixtures were assayed for NR activity after 0, 6, 10, 15 and 30 min incubation at 4°C.


Relative NADH-NR activity (%)
100
80
60
40
20
0
10
20
30
Time (min)

30 minutes at 4°C (Fig.38). The residual partial activities of NR after 0-10µl of anti-NR serum were incubated with 100µl purified NR for 30 min at 4°C are presented in Fig.39.

The NADH-, $FMNH_2$- and MVH-NR and NADH-CR activities of the NR preparation were all inhibited by greater than 3µl antiserum (Fig.39a). However up to 2µl antiserum were found to enhance (or stabilise) all of the NR activities compared with the control (0µl anti-NR serum). Pre-immune, control serum was also found to enhance NR activity (see later) and it appears that this non-specific effect of the antiserum overides the inhibitory effect of the NR antibodies up to 2µl anti-NR serum. The NADH-NR activity was most affected by this phenomenon, and the MVH-NR activity least affected. When results were expressed as a percentage of the 1µl antiserum value all of the NR activities were shown to be similarly inhibited (Fig.39b).

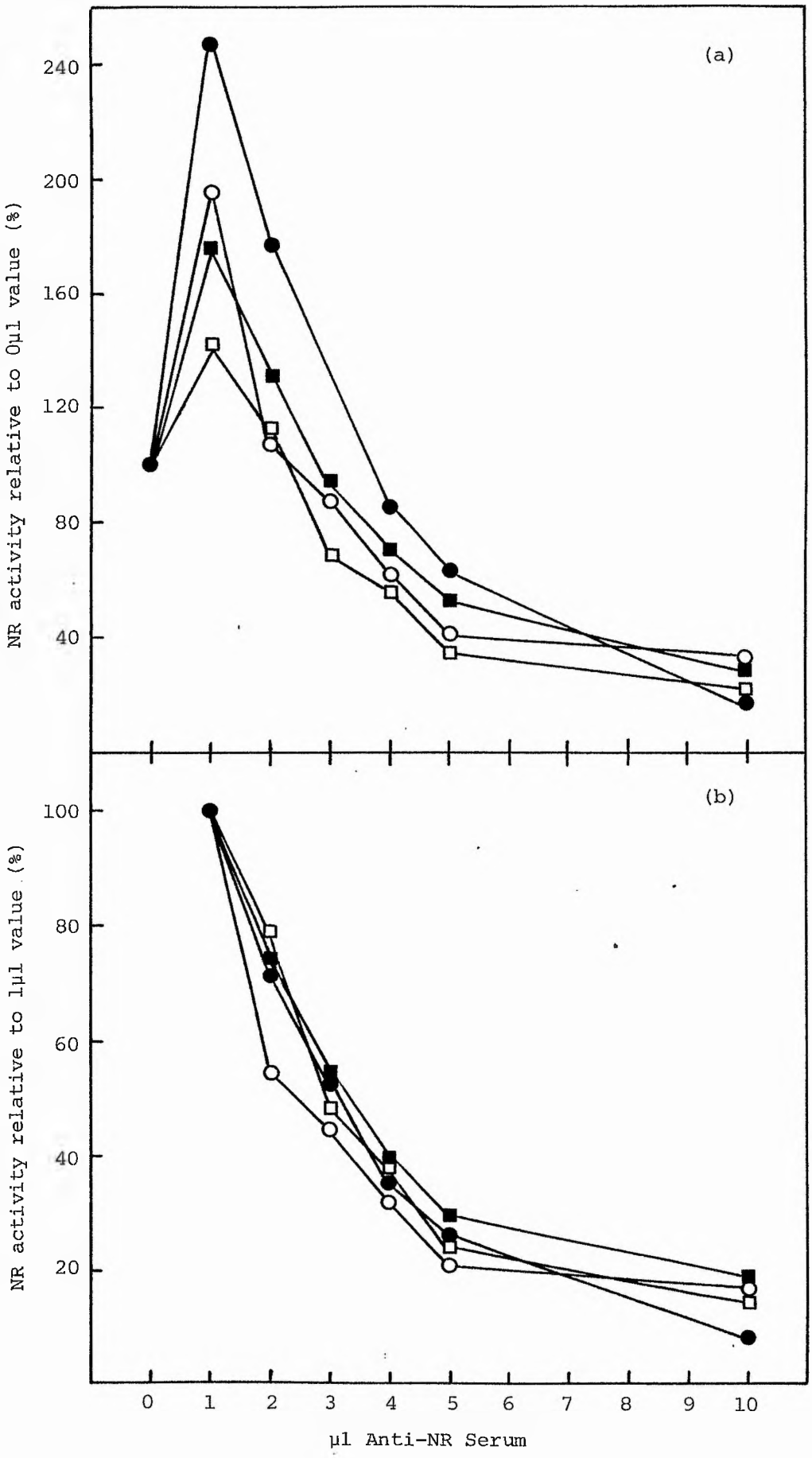
A lower than maximal titre sample of antiserum had been used for this investigation. When inhibition of NADH-NR only was carried out using the highest titre antiserum available this phenomenon was not observed (see Fig.40).

The results with barley resemble those obtained by Funkhouser and Ramadoss (1980) with antiserum against *C vulgaris* NR which was reported to inhibit totally all partial activities of the algal enzyme. Antiserum against squash NR (Smarrelli and Campbell, 1981) also inhibited all the activities of NR but the activities involving

Fig.39

Inhibition of the Partial Activities of NR by Anti-NR Serum

100µl of purified NR (1.33µmol nitrite produced/ml/h) were incubated with 0, 1, 2, 3, 4, 5 and 10µl of anti-NR serum in a final volume of 200µl with Buffer III. The mixtures were maintained at 4°C for 30 min after which time they were assayed for NADH-NR (●), $FMNH_2$-NR (○), MVH-NR (■) and NADH-CR (□) activities. Residual NR activity expressed as % of that in 0µl antiserum mixture (a) and 1µl antiserum mixture (b) were then calculated.


(a)
240
200
160
120
80
40
NR activity relative to 0µl value (%)
(b)
100
80
60
40
20
NR activity relative to 1µl value (%)
0
1
2
3
4
5
6
7
8
9
10
µl Anti-NR Serum

reduction of nitrate were inhibited to a greater extent than those which involved only the dehydrogenase function of the enzyme. Antiserum against *N. crassa* NR (Amy and Garrett, 1979) also inhibited all NR activites in a differential fashion.

These results indicate that the antiserum from all sources quoted contained antibodies to multi-antigenic sites on the NR molecule.

The Effect of Pre-Immune Control Serum on Purified NR

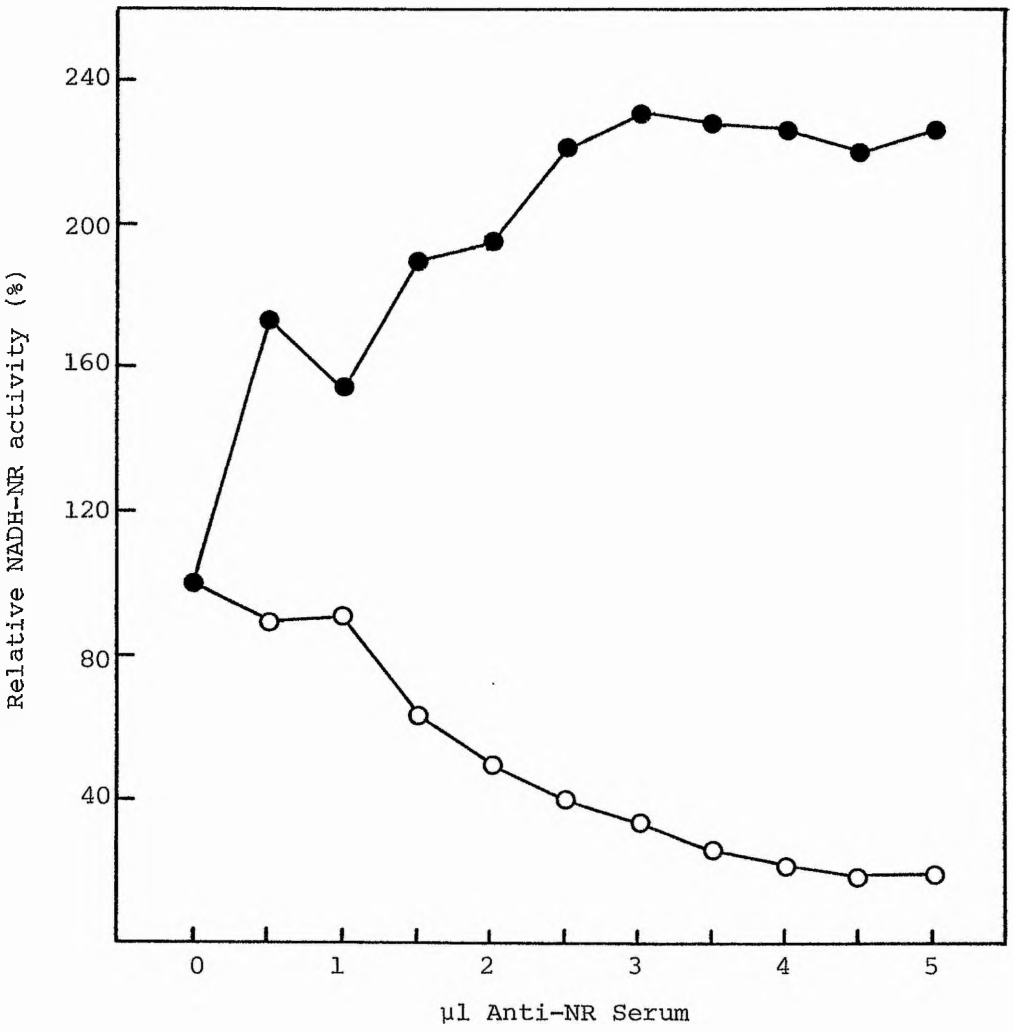
To illustrate the enhancing property of pre-immune serum on NR activity, purified NR was incubated with various amounts of pre-immune and immune serum as previously described (Fig.40). On this occasion the 'stimulatory' effect of pre-immune serum on NR activity increased up to 2.5µl of antiserum then reached a constant level at approximately 225% of the activity of the control incubation containing 0µl antiserum. 5µl of this, the highest titre sample of antiserum available, inactivated the majority of NR activity in the preparation while the enhancement of NR activity with low levels of anti-NR serum shown previously with inferior antiserum was not observed.

The enhancement of NR activity by pre-immune serum has been observed in all systems studied (higher plant, algal and fungal) albeit to different degrees.

Fig.40

Effect of Pre-Immune Serum on Purified NR

100µl of purified NR (2.01µmol nitrite produced/ml/h) were incubated with 0-5µl of pre-immune serum (●) and 0-5µl of immune serum (o) in final volumes of 200µl with Buffer III. The mixtures were maintained at 4°C for 30 min after which time they were assayed for NR activity.


240
200
160
120
80
40
Relative NADH-NR activity (%)
0
1
2
3
4
5
µl Anti-NR Serum

Since BSA has been shown to stabilise NR in crude and purified NR preparations (this thesis) albumin was removed from the crude antisera (pre-immune and immune) in order to determine whether it had any effect on ability to enhance NR activity.

The protein in 1 ml of pre-immune serum and 1 ml of anti-NR serum was precipitated with an equal volume of saturated $(NH_4)_2SO_4$, pH 7.5. Precipitated proteins were collected by centrifugation and the pellet washed at 4 °C with a solution of 1.75M $(NH_4)_2SO_4$ until white. This procedure removes albumin, transferrin, and α proteins including haptoglobin and haemoglobin (Mayer and Walker, 1978). The remaining protein was then redissolved in 1 ml Buffer III. The semi-purified samples of immune and pre-immune serum were assessed for their effect on NR activity along with untreated immune and pre-immune serum using the assay described previously (Fig.41).

The semi-purified pre-immune serum maintained its ability to enhance NR activity up to 2µl serum which then dropped off dramatically in an inexplicable fashion. The titre of the purified immune serum did not appear to be affected significantly by the removal of albumin. It appears that the increase in NR activity in the presence of pre-immune serum cannot be wholly attributed to the presence of albumin.

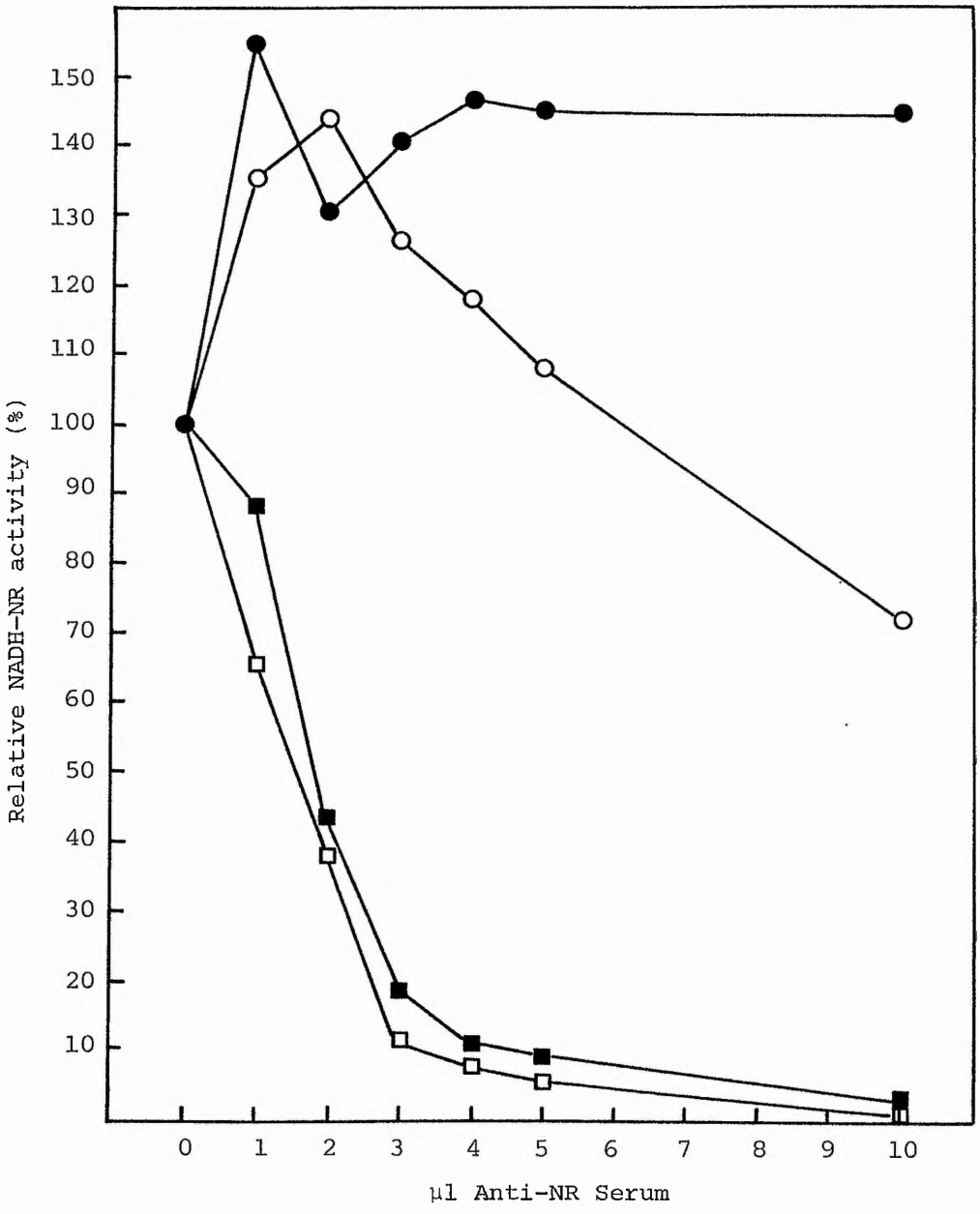
Graf *et al* (1975) found a similar phenomenon with spinach NR and suggested that these stimulatory effects

Fig.41

To Assess the Contribution of Albumin on the Effect of Pre-Immune Serum on NR Activity

Albumin, along with other serum proteins, was removed from aliquots of immune and pre-immune serum as described in the text. The remaining protein was then redissolved in Buffer III to give comparable volumes with aliquots of untreated immune and pre-immune serum.

100µl of purified NR (1.14µmol nitrite produced/ml/h) were incubated with 0, 1, 2, 3, 4, 5 and 10µl of crude pre-immune serum (●), crude anti-NR serum (■), partially purified pre-immune serum (○) and partially purified immune serum (□) in final volumes of 200µl with Buffer III. The mixtures were maintained at 4°C for 30 min after which time they were assayed for NADH-NR activity.


Relative NADH-NR activity (%)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
µl Anti-NR Serum

were due to secondary effects rather than a direct protection of NR from decay by the serum proteins.

Inhibition of Barley NR and Associated Activities by Antiserum Directed Against the 40 000 MW NADH-CR Species

Antiserum directed against the 40 000 MW NADH-CR species inhibited Blue Dextran Sepharose purified 40 000 MW NADH-CR species (see legend to Fig.37b for details).

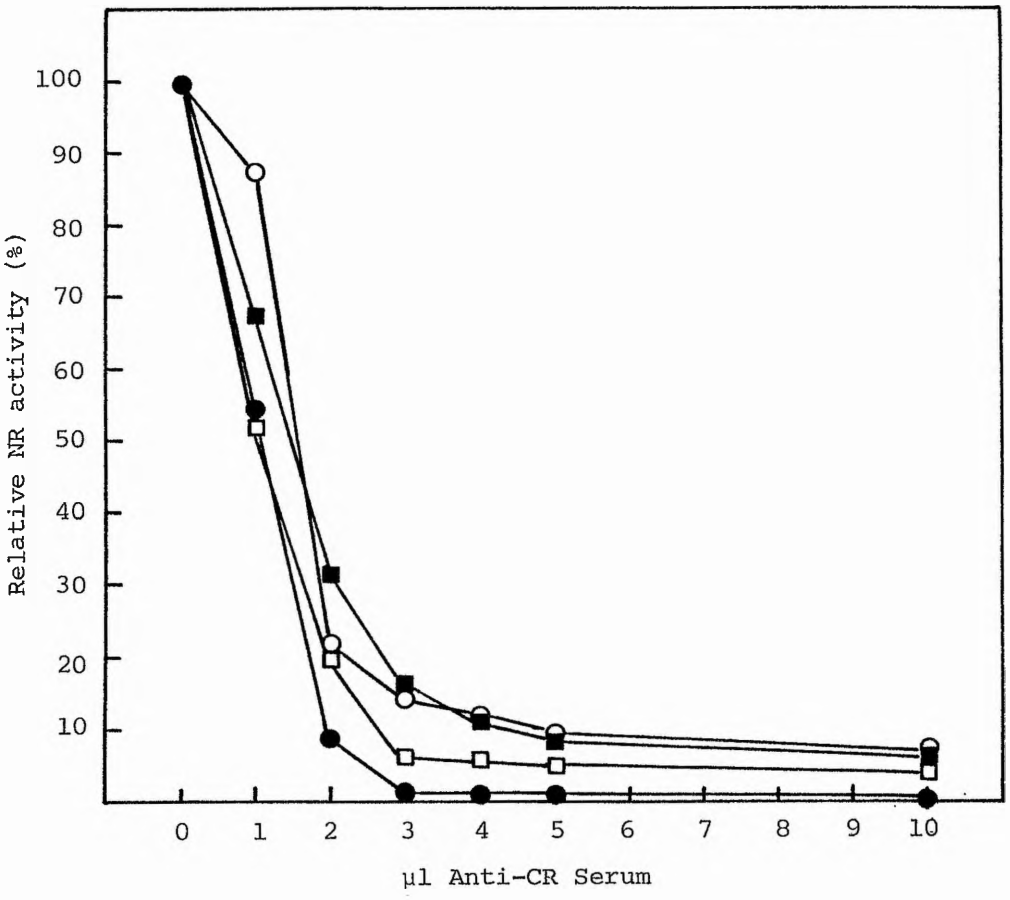
Inhibition of NADH-CR activity in a preparation of 40 000 MW CR species by anti-NR serum was not investigated here but has subsequently been shown to occur (J. McA. Campbell, personal communication).

The residual partial activites of nitrate reductase after 0-10µl of anti-NADH-CR species serum were incubated with 100µl of Blue Dextran Sepharose purified NR for 30 min at 4°C are presented in Fig.42. The NADH-, $FMNH_2$- and MVH- nitrate reductase and NADH-CR activities were similarly inhibited by the anti-40 000 MW NADH-CR species serum and the implications of these observations will be discussed in the General Discussion section. However, it appears that the antiserum directed against this nitrate reductase related NADH-CR species is more effective in inhibiting NR activities than antiserum directed against NR itself.

Fig.42

Inhibition of Partial Activities of NR by Anti-40 000 MW NADH-CR Species Serum

100µl purified NR (0.69µmol nitrite produced/ml/h) were incubated with 0-10µl of anti-40 000 MW NADH-CR species serum in a final volume of 200µl with Buffer III and maintained at 4 °C. After 30 min the mixtures were assayed for NADH-NR (●), $FMNH_2$-NR (○), MVH-NR (■) and NADH-CR (□) activity.


Relative NR activity (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
µl Anti-CR Serum

Estimation of Cross Reacting Material (CRM) by use of a Protection of Inhibition Assay

The protection of inhibition assay, in various forms, has proved useful in semi-quantitating NR protein from *N. crassa* (Amy and Garrett, 1979), spinach (Graf *et al*, 1975) and barley (Kuo *et al*, 1981). The assay is suitable for use with antiserum which is not monospecific since it provides a method for detecting the presence of CRM that is independent of the ability of the antibody to precipitate the protein.

In this assay fixed amounts of NR protein (standard NR preparation) and antiserum are mixed together and the amount of inhibition assessed. Plant extracts of interest are then incubated with identical mixtures of NR and antiserum. If additional cross reacting antigens are present in the extract then the inhibition of NR is less.

Development of a Protection of Inhibition Assay

Preliminary experiments using a fixed amount of antiserum proved insensitive therefore it was decided to employ varying amounts of antiserum and the amount of antiserum which caused 50% inactivation of NR activity used as an indication of the level of CRM. At 50% inactivation the more linear relationship would presumably produce more quantitative estimates of CRM. This approach has been used previously by Graf *et al* (1975) when estimating NR-CRM in spinach leaf extracts.

Initially Blue Dextran Sepharose purified NR was used as the source of standard NR protein in the assay but the highly unstable nature of this preparation resulted in poor reproducibility. Cell-free extract of 90 h old plants was therefore used since NR has been shown to be relatively stable in this preparation. The cell-free extract was prepared as described in Methods, Section II except that a buffer : g FW ratio of 4:1 was employed and filtered extracts were centrifuged for 20 min at 4°C and 100 000 g (40 000 rpm) in a Prepsin 50 centrifuge.

Cell-free extracts of nitrate-less and ammonium-grown plants were prepared in the same way from barley plants which had been watered daily with half-Hoagland nutrient solution lacking nitrate and ammonium and nutrient solution containing 15mM NH_4Cl respectively.

Estimation of Inactivation Strength of Anti-NR Serum

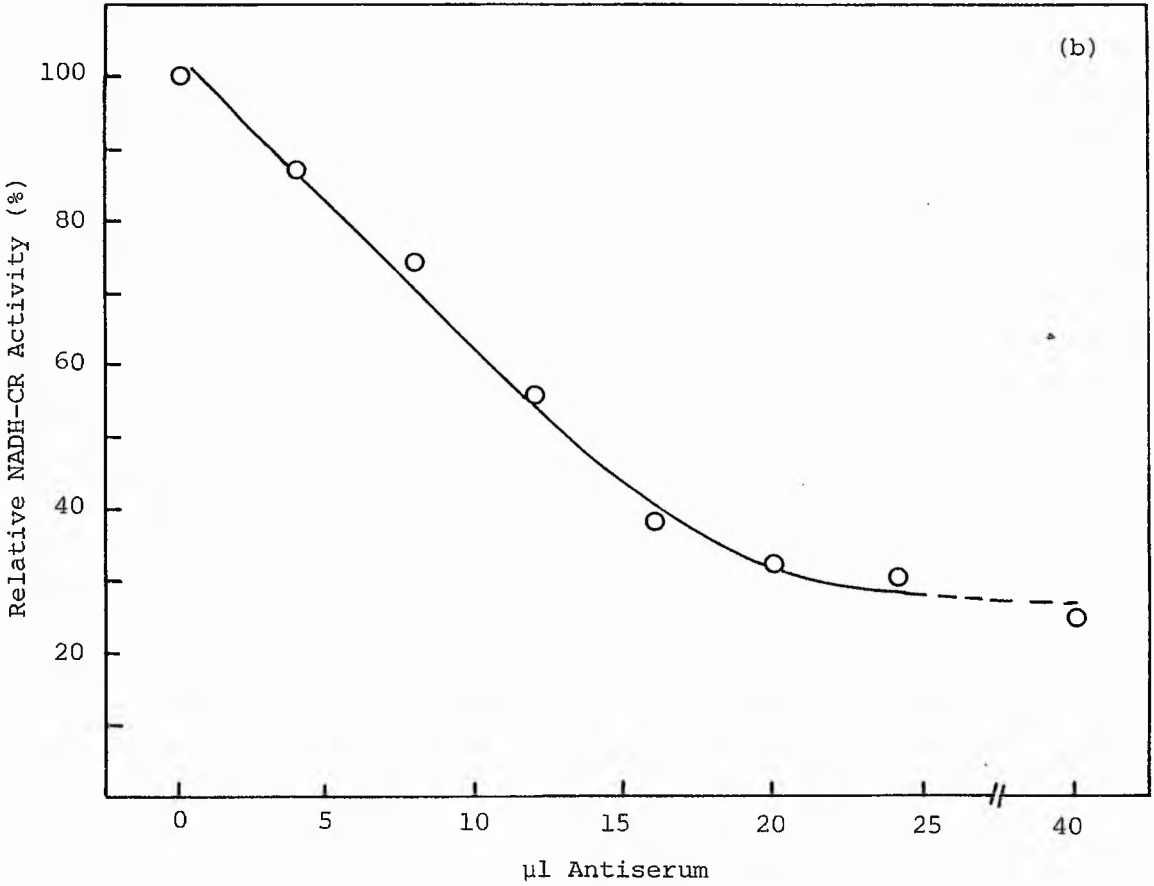
The strength of the antiserum raised against purified NR was assessed by its ability to inhibit NR activity in cell-free extracts of 90 h old nitrate-grown plants (Fig.43a).

On this occasion an approximately linear relationship between NR inactivation and amount of antiserum added was observed up to 5µl of antiserum in the assay. The first 5µl of antiserum inactivated 70% of the NR activity but at least another 10µl were required to inactivate the remaining 30% (the exact amount being difficult to determine).

Fig.43

Estimation of Inactivation Strength of Anti-NR Serum

Various amounts of anti-NR serum made up to a final volume of 50µl with Buffer I were mixed with 250µl Buffer I in a 1.5 ml Eppendorf tube and maintained at 4 °C for 45 min. 250µl of cell free extract derived from nitrate-grown plants (control NR protein) were then added (giving a final volume of 550µl) and maintained at 4 °C for a further 45 min. 45 min had been previously found to be sufficient time for inactivation to reach end point (See Fig.43a). The mixtures were then centrifuged in an Eppendorf bench top centrifuge at full speed for 10 min after which time the supernatants were immediately assayed for NADH-NR (a) and NADH-CR (b) activities. The activities obtained in the absence of added antiserum served as the control.


(b)
Relative NADH-CR Activity (%)
100
80
60
40
20
0
5
10
15
20
25
40
µl Antiserum

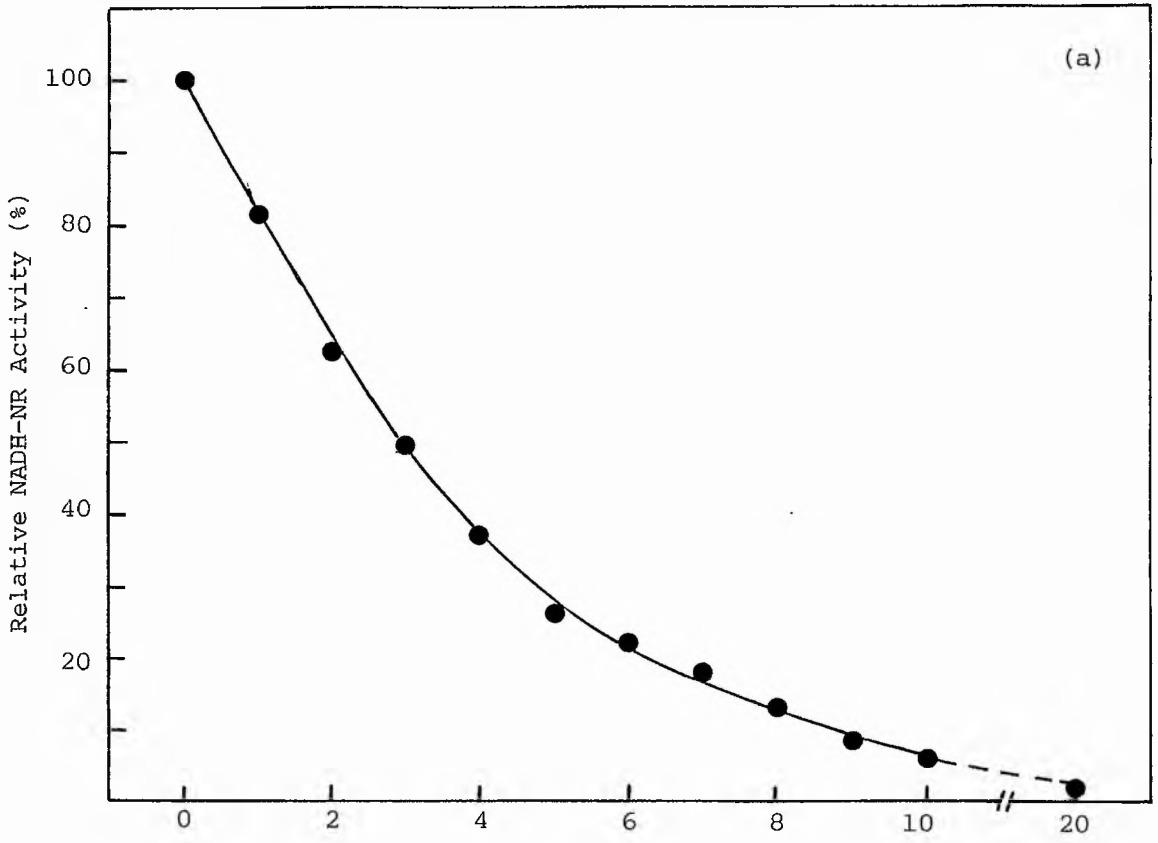
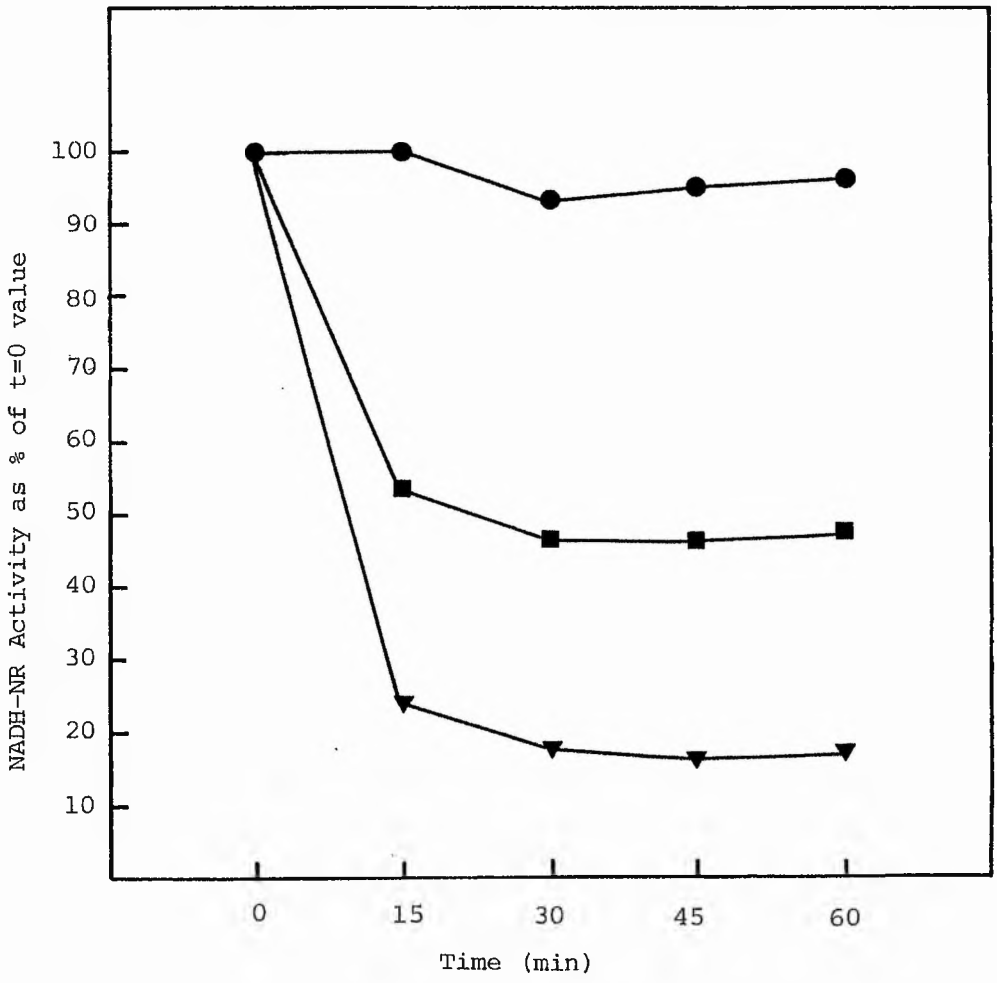

(a)
Relative NADH-NR Activity (%)
100
80
60
40
20
0
2
4
6
8
10
20

Fig.43a

Determination of a Suitable Incubation Time for Inhibition of NR by Anti-NR Serum Employing Cell-Free Barley Leaf Extract as the Source of NR

0 (●), 5 (■) and 10 (▼) µl of anti-NR serum, made up to a final volume of 50µl with Buffer I, were mixed with 250µl of Buffer I and 250µl of cell-free extract, derived from nitrate-grown plants, in 1.5 ml Eppendorf tubes. The mixtures were maintained at 4°C for 0, 15, 30, 45 and 60 min after which time they were centrifuged in an Eppendorf bench-top centrifuge at full speed for 10 min. The supernatants were then immediately assayed for NADH-NR activity.


NADH-NR Activity as % of t=0 value
100
90
80
70
60
50
40
30
20
10
0
15
30
45
60
Time (min)

In contrast, even at very high concentrations (up to 40µl antiserum), 25% of the NADH-CR activity in the cell-free extract remained active (Fig.43b). This resistant CR activity presumably comprised the constitutive CR species known to be present in cell free extracts which are not related to NR.

In 7 separate experiments using plants which were 90-96 h old a mean of 4.9µl of antiserum were required to inactivate 50% of the NR activity in nitrate cell-free extract NR activity of the extract (range 6.04 - 7.76µmol nitrite produced / g FW/h) and µl antiserum required to inactivate 50% of the NR activity (range 2.9 - 6.6µl) were not correlated (Table 19).

Effect of Plant Age on the Amount of Anti-NR Serum Required to Inactivate 50% of NR Activity in Cell-Free Extracts of Nitrate-Grown Plants

To determine whether age of the plant material contributed to this observed variability the ability of anti-NR serum to inactivate NR in cell free extracts of 5 day old primary leaves was assessed as previously described for 4 day old leaf extracts.

A mean of 5.8µl of antiserum were required to inactivate 50% of the NR activity in cell free extracts derived from older tissue (114-125 h old) in 6 separate experiments. NR activity of the extracts (range 2.59 - 5.10µmol nitrite produced/g FW/h) and µl antiserum required to inactivate 50% of the NR activity (range 4.5 -

8.0) were not correlated (Table 19).

It does appear, however, that the mean amount of antiserum required to inactivate 50% of NR activity is higher for the older tissue derived extract despite the mean NR activity being lower. It has previously been noted in this thesis (Chapter 2) that NR activity in older tissue extracts is labile and breaks down into smaller CR species. It seems, therefore, that the anti-NR serum may also react with degradation products of NR in these older leaf extracts.

However, even if plant age does contribute to the variability observed in the inhibition assay one might expect the effect over 4 h to be minimal but 3 separate extractions performed when plants were 90, 91 and 94 h old required 3.5, 3.1 and 6.4µl antiserum respectively.

It is more likely that variation in the extraction efficiency of the leaf tissue is a greater contributory factor. Unfortunately the specific activity of NR in the cell free extracts was not determined to test this hypothesis.

The establishment of a standard curve for each protection of inhibition assay is thus of great importance.

Table 19 Estimation of Antigenicity and NR Activity in Cell-Free Extracts of Nitrate Grown Plants

Plant age (h)	NR activity (µmol NO_2^- produced/ ml extract/h)	Antiserum required for 50% inactivation of NR (µl)
90-96	6.11	2.9
	6.76	3.5
	7.76	3.1
	6.04	6.4
	6.76	6.0
	7.61	6.0
	7.06	6.6
114-125	4.17	5.1
	4.74	5.3
	5.03	4.5
	4.88	4.7
	5.10	8.0
	2.59	6.9

Quantitation of the Protection of Inhibition Assay

To estimate the amount of CRM in cell free extracts of interest an identical procedure to that described for assessment of NR inactivation strength was adopted except that various amounts of antiserum were first reacted with 250µl cell free extract for 45 min at 4 °C. Controls were made in the same way but contained no antiserum.

To permit a direct comparison between assays a standard curve was established for each experiment under identical conditions by allowing the standard NR protein preparation to react with different amounts of antiserum.

Any extract in the inactivation assay which requires more antiserum than the amount required to inactivate 50% of the standard NR protein must contain NR cross reacting antigens, and the volume of antiserum indicates the degree of antigenicity in the cell free extract of interest.

Kuo (1979) described a method for the semiquantitation of the level of CRM, the values of which were dependent upon the end point of the titration of NR in cell free extract (see below for worked example). Since the end point is difficult to establish, the values of amount of antigen are therefore only estimates.

To assess the linearity of the assay and to test the Kuo method of quantitation a cell free extract from nitrate-grown plants was diluted 0:1, 1:3, 1:1 and 1:0 with Buffer I. These dilutions, which were theoretically equivalent to extracts containing 0, 25, 50 and 100% CRM

respectively, were subjected to the protection of inhibition assay as previously described.

From the standard NR protein inactivation curve (Fig.44 □-□) it can be seen that approximately 20µl were required to completely inactivate NR activity. 6µl were required to inactivate the first 50% of activity therefore it follows that approximately 14µl were needed to inactivate the 2nd 50% of activity. Thus for the dilution of extract which theoretically contained 50% CRM (Fig.44 o-o) out of the 14µl of antiserum required to result in a 50% inactivation 14 - 6 = 8µl were presumably neutralised by NR-CRM in the diluted extract allowing the rest of the 6µl of antiserum to inactivate 50% of added NR in the reaction mixture. The estimated level of antigenicity in this extract is therefore 8/14 or approximately 57% of the standard NR preparation. Similarly the levels of antigenicity in the extracts diluted to give theoretically 25 and 100% CRM were 4.6/14 = 32.9% and 10/14 = 71.4% respectively.

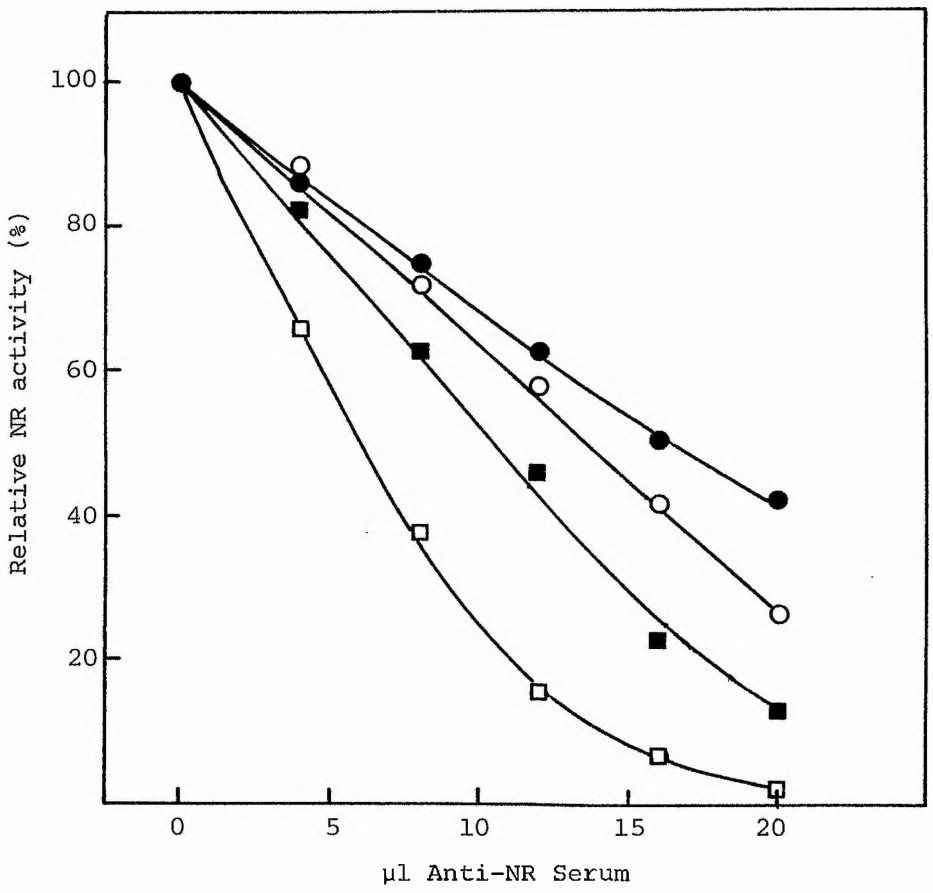
It would appear that this protection of inhibition assay is linear to approximately 50% CRM. However, if CRM in the extract of interest did not also contribute to NR activity of the mixture (e.g. mutant extract) then this may not be the case since Kuo (1979) detected antigenicity exceeding the level of the wild type (approximately 140% for Az28 and 125% for Az32).

Fig.44

Linearity of the Protection of Inhibition Assay

A cell-free extract from 90 h old nitrate-grown plants was diluted 0:1, 1:3, 1:1 and 1:0 with Buffer I to give extracts which theoretically contained 0, 25, 50 and 100% NR-CRM respectively.

0, 4, 8, 12, 16 and 20µl of anti-NR serum (in a final volume of 50µl with Buffer I) were mixed with 250µl of the extracts representing 0% (□), 25% (■), 50% (○) and 100% (●) CRM in a 1.5 ml Eppendorf tube and maintained at 4 °C for 45 min. 250µl of nitrate cell-free extract (control NR protein) were then added (giving a final volume of 550µl) and maintained at 4 °C for a further 45 min. The mixtures were then centrifuged and the supernatants assayed for NR activity.


100
80
60
40
20
Relative NR activity (%)
0
5
10
15
20
µl Anti-NR Serum

Semiquantitative Estimation of CRM in Non-Induced and Ammonium-Grown Cell-Free Extracts

Five separate experiments were performed to determine whether CRM was present in non-induced and ammonium-grown cell-free extracts (Table 20). Using the method of Kuo (1979) to quantitate the amount of CRM it would appear that non-induced cell-free extracts contained negligible NR-CRM (mean 7.8%) while ammonium-grown cell free extracts appear to contain a significant amount of CRM (mean 22.4%).

However, it must be noted that, a small amount of NR activity (mean 13.7% of an equivalently aged cell-free extract derived from nitrate-grown plants) could always be detected in ammonium-grown cell-free extracts. Whether the difference between CRM and NR activity is significant is not clear. Since the plants were not grown in sterile conditions it is possible that the plants may have indirectly received nitrate.

Kuo in preliminary work with antigenicity of barley mutants (1979) showed also that seedlings grown without nitrate had very low levels of NR-CRM but ammonium-grown plants were not investigated at that time.

It has been shown by various immunological techniques that, in *Chlorella vulgaris*, ammonium-grown cells which contain almost no active enzyme still contain material which cross reacts with antibodies to NR (Funkhouser and Ramadoss, 1980). In contrast Amy and Garret (1979) using similar immunological techniques showed that ammonium-

Table 20 Estimation of Antigenicity of Non-Induced and Ammonium-Grown Cell-Free Extracts Using the Protection of Inhibition Assay

Expt	Antiserum required for 50% inactivation of NR (µl)			Antiserum required for 100% inactivation of added NR (µl)	Estimated CRM (%)	
	NO_3 induced	Non-induced	Ammonium grown		Non-induced	Ammonium grown
1	2.9	4.7	7.0	15	14.9	33.9
2	3.5	3.5	5.1	16	0	12.8
3	3.5	4.4	7.1	16	7.2	28.8
4	6.4	10.4	11.6	30	16.9	22.0
5	6.0	5.05	8.8	25	-0.1	14.7
$\bar{x}$	4.38	5.61	7.92	19.4	7.8	22.4

grown mycelia contained no NR-CRM. The presence or absence of CRM in ammonium-grown cells is of importance in determining whether subsequent nitrate induction is due to activation of a pre-existing NR (as in *Chlorella*) or protein synthesis (as in *Neurospora*).

Effect of Nitrate Addition on NR Activity and NR-CRM in Cell-Free Extracts of Non-Induced and Ammonium-Grown Barley Plants

Since the level of NR-CRM in this study has been equivocal it was decided to investigate the nitrate induction of nitrate-less and ammonium-grown plants to determine whether any further information could be gained from comparing the response of CRM and NR activity to added nitrate (Figs.45 and 46 respectively).

Percentage CRM in the control, uninduced treatment (Fig.45) correlated more closely with NR activity than in the control ammonium-grown treatment.

On nitrate induction of both treatments it would appear that there is a lag period in which NR activity of the extracts does not increase while percentage CRM markedly increases in the previously nitrate-less plants and maintains a high level in the previously ammonium-grown plants.

After 24 h, percentage CRM and NR activity corresponded well. It is not however clear whether this is a true

Fig.45

Nitrate Induction of NR in Previously Nitrate-Less Barley Plants

Barley seedlings were grown as previously described and watered daily with half-Hoagland nutrient solution lacking nitrate. After 4 days (at t = 0) one tray of seedlings was watered with half-Hoagland nutrient solution (containing 15mM KNO_3). At t = 0, 1, 4 and 24 h leaves were harvested from the induced and non-induced seedlings and cell-free extracts prepared as described in the text. NR activity and NR-CRM were estimated as previously described.

NR activity (non-induced (■) and induced (□)) and NR-CRM (non-induced (●) and induced (○)) were expressed as a percentage of results obtained from extracts derived from equivalently aged nitrate-grown plants.

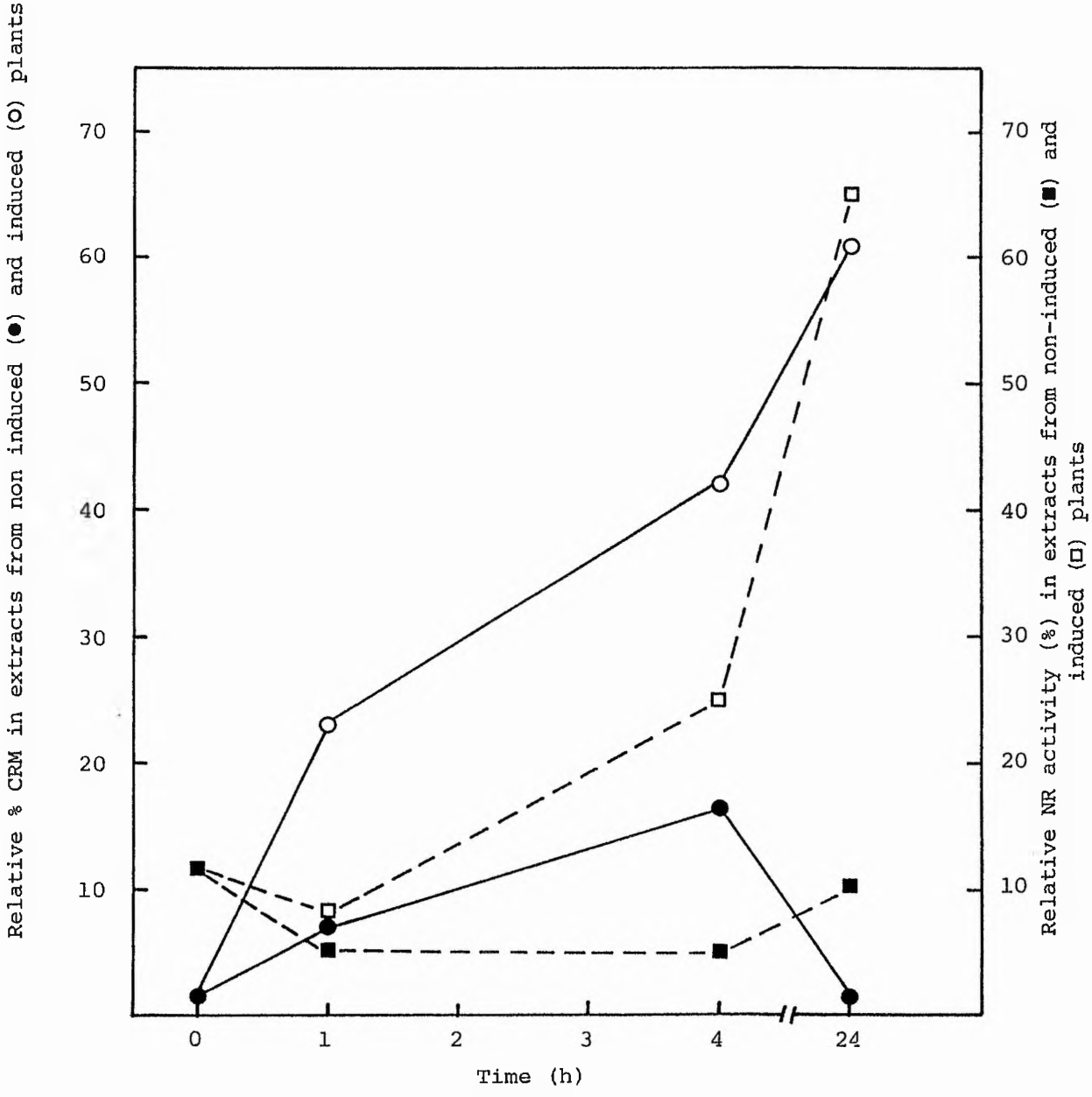
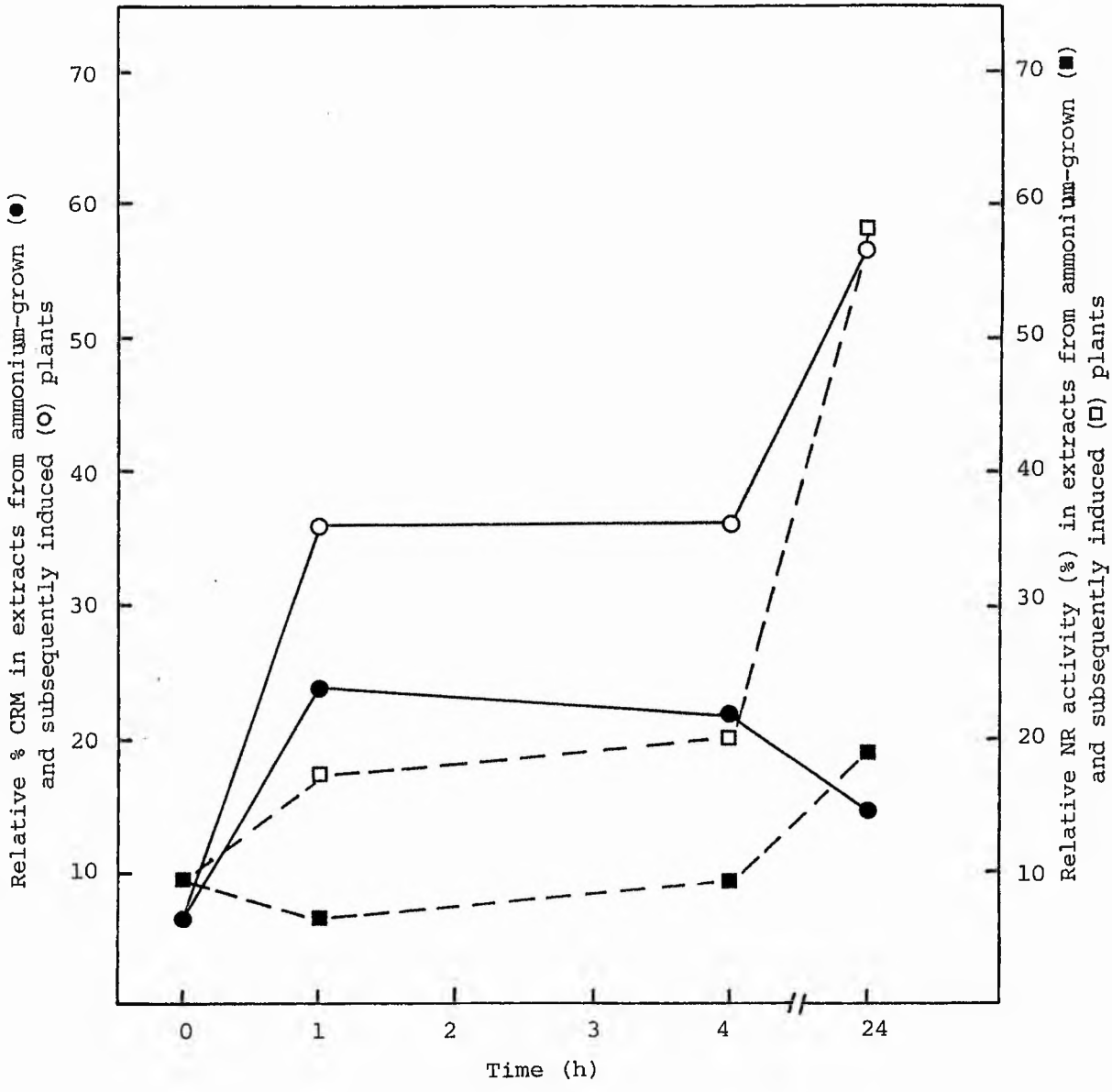

Relative % CRM in extracts from non induced (●) and induced (○) plants
Relative NR activity (%) in extracts from non-induced (■) and induced (□) plants
70
60
50
40
30
20
10
0
1
2
3
4
24
Time (h)

Fig.46

Nitrate Induction of NR in Previously Ammonium-Grown Barley Plants

Barley seedlings were grown as previously described and watered daily with half-Hoagland nutrient solution lacking KNO_3 and containing 15mM NH_4Cl. After 4 days (at t = 0) one tray of seedlings was watered with half-Hoagland nutrient solution. At t = 0, 1, 4 and 24 h leaves were harvested from the induced and non-induced seedlings and cell-free extracts prepared as described in the text. NR activity and NR-CRM were estimated as previously described.

NR activity (non-induced (■) and induced (□)) and NR-CRM (non-induced (●) and induced (○)) were expressed as a percentage of results obtained from extracts derived from equivalently aged nitrate-grown plants.


Relative % CRM in extracts from ammonium-grown (●) and subsequently induced (○) plants
Relative NR activity (%) in extracts from ammonium-grown (■) and subsequently induced (□) plants
Time (h)
0
1
2
3
4
24
10
20
30
40
50
60
70

reflection of the situation or whether it is due to the non-linearity of the protection of inhibition assay above 50% CRM resulting in a lower percentage CRM.

It will be noted that even after 24 h only approximately 60% of the NR activity present in the induced control was attained in both the nitrate-less and ammonium-grown plants induced with nitrate after 4 days growth. It would appear, therefore, that continuous presence of nitrate is required for maximal NR activity.

Unfortunately this experiment was not repeated and it is clear that no conclusions regarding the mechanism of nitrate induction can be drawn from results such as these. However, it would appear that during the lag phase of induction of active NR, precursor NR protein is synthesised in previously nitrate-less plants and already present in ammonium-grown plants which is capable of cross reacting with anti-NR serum.

Investigation into the Presence of Non-Specific 'CRM' in Nitrate-Less and Ammonium-Grown Cell-Free Extracts

Kuo (1979) showed that when non-induced seedlings were homogenised in Tris buffer alone, there may have been interfering substances present in the cell-free extracts which non-specifically interacted with antiserum. He found that by adding 3% BSA to the extraction buffer the effect of interfering substances was minimised. It was suggested that these interfering substances might be

polyphenolics which have been shown to be effectively absorbed by BSA in cell-free extracts (Loomis, 1974; Siegel and Enns, 1979).

112 h old induced, non-induced and ammonium-grown plants were extracted in Buffer I containing no additions or Buffer I containing 3% BSA. Cell free extracts were prepared as previously described and were subjected to the protection of inhibition assay using the extracts (plus and minus BSA) derived from the nitrate induced plants as NR control protein as appropriate (Table 21).

Presence of BSA during extraction has previously been shown to stabilise NR in older plant tissue. From the results presented in Table 21 it would appear that it is also capable of doing so where negligible NR activity is present as in uninduced and ammonium-grown plant extract. This is reflected in the apparently greater amount of antiserum required to inactivate 50% of added NR activity when BSA is present.

Since both extracts, with and without BSA, derived from non-induced plants resulted in approximately 0% CRM on this occasion no conclusions of significance can be drawn re the effect of BSA.

An alternative explanation for the greater amount of antiserum required to inactivate NR in the BSA treatments might be that BSA interferes with the binding of antibodies to NR thus offering protection of inhibition either by

Table 21 NR Activity and NR-CRM in Cell-Free Extracts of 112 h old Non-Induced and Ammonium-Grown Plants Prepared in the Presence and Absence of 3% BSA

BSA	Initial NR activity of extract (µmol nitrite produced/ml extract/h)			Antiserum required for 50% inactivation of NR (µl)			Estimated NR-CRM (%)	
	nitrate-grown	non induced	ammonium grown	nitrate-grown	non induced	ammonium grown	non induced	ammonium grown
-	5.10	*ND	0.52	4.5	4.7	>10	1.5	-
+	5.38	0.10	1.06	6.3	5.9	>10	-2.0	-

*ND = none detectable

binding to the antibodies, by binding to NR or spacially separating the antibodies from NR.

Effect of NADH and Nitrate on Inhibition of NR by Anti-NR Serum

Pre-treatment of nitrate induced cell free extract and anti-NR serum with nitrate and with NADH was investigated to determine whether excess substrate and cofactor of NR were capable of interfering with the binding of antiserum to NR thus offering protection of inhibition of NR against anti-NR antibodies.

Kinetic properties of higher plant NR indicate a K_m for nitrate and NADH of 200µM and 10µM respectively therefore final concentrations of 2.0mM and 0.1mM were used for preincubation, being equivalent to ten times the K_m values.

(a) Pre-treatment of Antiserum with 0.1mM NADH and 2.0mM KNO_3

Various amounts of anti-NR serum were incubated in 1.5 ml Eppendorf tubes with: 250µl Buffer I; 250µl Buffer I containing 0.1mM NADH; 250µl Buffer I containing 2.0mM KNO_3 in a final volume of 300µl for 45 min at 4°C. 250µl cell-free extract from nitrate induced plants (extraction ratio, 1:4 gFW:Buffer) were then added and the mixtures incubated for a further 45 min at 4°C. The incubates were then centrifuged and the supernatants

immediately assayed for NR activity.

(b) Pre-treatment of NR with 0.1mM NADH and 2.0mM KNO_3

Cell free extract derived from nitrate induced plants (extraction ratio, 1:3 gFW:Buffer) was diluted 3:1 with Buffer I; Buffer I containing 0.4mM NADH (giving final concentration of 0.1mM NADH in the mixture); Buffer I containing 8mM KNO_3 (giving final concentration of 2mM KNO_3 in the mixture). The NR preparations were maintained at 4°C for 45 min during which time various amounts of antiserum were incubated in Eppendorf tubes with 250µl Buffer I in a final volume of 300µl.

The pre-incubated NR preparations were then added to the antisera and incubated for a further 45 min at 4°C. The mixtures were then centrifuged and the supernatants assayed immediately for NR activity.

Controls were set up in the same way but contained no antiserum.

The amount of antiserum required to inactivate 50% of the added NR activity plus the NR activity remaining in the control incubates for each treatment are shown in Table 22.

It should be noted that blank assays for NR activity were not performed in this experiment. The greater amount of activity observed in the NADH treatment is therefore most likely to have been a result of nitrite

Table 22 Effect of NADH and Nitrate on Inhibition of NR Activity by Anti-NR Serum

Treatment	Amount of antiserum required to inactivate 50% of the NR activity (µl)	NR activity in mixtures containing no antiserum (µmoles NO_2^- produced/ h/g FW)
Antiserum pre-incubated with		
Buffer I	5.1	5.32
Buffer I + NADH	6.0	9.61
Buffer I + NO_3	5.2	6.08
NR pre-incubated with		
Buffer I	5.3	5.89
Buffer I + NADH	6.0	8.82
Buffer I + NO_3	4.9	6.47

formed during incubation prior to assay due to endogenous nitrate present in the extract. Presumably there was less endogenous NADH in the extract since pre-incubation with nitrate increased apparent NR activity to a lesser extent.

Even on assuming that the amount of NR protein is grossly overestimated by the NR activity in the NADH and nitrate treatments it would still appear from the amount of antiserum required to inactivate 50% of NR activity that pre-treatment of either NR or antibody with NADH, but not with nitrate, preserves the enzyme to some extent from antibody inhibition. Whether this was due to NADH confering stability on NR or that changes in NR structure on reduction with NADH results in changes in antigenic sites, is not clear.

GENERAL DISCUSSION

The aims of this General Discussion section are: to draw together the evidence for *in vitro* degradation of NR; to examine the implications of this with respect to the structure of higher plant NR and to assess its significance with respect to turnover and regulation of NR *in vivo*.

SECTION I NATURE OF THE NADH-CR SPECIES IN BARLEY LEAF EXTRACTS

As described in Chapter 2 of the Results, there are 3 major NADH-CR species which are believed to be derived from barley NR. These species have calculated molecular weights of 40 000 (3.1S), 61 000 (3.8S) and 163 000 (6.8S), the largest of the species also exhibiting NADH-NR activity. There appears to be a general consensus that the NADH-NR of higher plants (MW approximately 200 000) consists of two haemoflavoprotein subunits (MW approximately 100 000) and a low MW MoCo (see Introduction). If the NADH-CR species observed in barley leaf extracts represent intact subunits and associations of subunits, then it is clear that the proposed dimeric structure of the enzyme is not correct. It is therefore crucial to establish whether these CR species are derived from NR and if so, establish a mechanism by which they may be generated.

In an attempt to obtain direct evidence that at least one of these species, namely the 40 000 MW CR

species, is related to NR, antibodies to purified NR and purified MW 40 000 CR species were raised (Chapter 7). Antibodies raised against the 40 000 MW CR species were found to inhibit all NR-associated activities (Chapter 7 and J. McA. Campbell, personal communication) and antibodies raised against NR inhibited the NADH-CR activity of a purified 40 000 MW CR species preparation (J. McA. Campbell, personal communication). Although it was not possible to assess the specificity of the antisera described in this thesis, J. McA. Campbell working in the same laboratory concluded that the antigens used by him to raise antibodies were not contaminated and thus one can conclude that the 40 000 MW CR species, at least, is related NR.

Various pieces of circumstantial evidence endorse this conclusion, in addition to providing evidence that the other species are also related to NR. The MW 163 000, 61 000 and 40 000 NADH-CR species are present only in cell-free extracts from plants treated with nitrate. If these species are unrelated to NR, then they are "induced" by nitrate together with NR (Small and Wray, 1981a).

Sucrose density gradient analysis of an aliquot of Biogel A 1.5 m eluent which would be expected to contain no small CR species revealed the presence of relatively large amount of 3.8 and 3.1S CR species which would appear to have been released from unstable NR molecules (Small and Wray, 1981a).

Loss of the 7.7S CR species (intact NR complex) which occurs in cell-free extracts prepared from shoots older than 4 day is accompanied by the appearance of increased amounts of these small CR species (Chapter 2, Fig.11). Further, inclusion of BSA in the extraction buffer was shown not only to prevent loss of the 7.7S CR species but also to prevent formation of increased amounts of the small CR species (Chapter 2, Fig.13).

Mechanisms by which BSA protects NR from this age-dependent inactivation *in vitro* have been discussed in detail in Chapter 2 of the Results. BSA does not appear to stabilise NR through increasing protein concentration *per se* and thus preventing dissociation of the NR molecule (Chapter 2, Tables 8 and 9 and Fig.15) but is thought more likely to operate by protecting NR from an inactivating mechanism which became increasingly active with age. Since the proteinase inhibitors PMSF (Chapter 2, Table 11 and Fig.16) and 1,10-phenanthroline (Chapter 2, Table 13) retard the loss of NR activity in older tissue it is likely that the inactivating system comprises a proteolytic component. Support for this comes from the observation (Chapter 5, Fig.32) that trypsin cleaves purified NR into CR species with the same sedimentation coefficients as those observed in crude extracts. As BSA also retards this conversion (Chapter 5, Fig.34) it is possible that the non-plant protein acts as an alternative substrate for trypsin.

At the time this work was carried out there was no direct evidence for an NR specific proteinase in barley leaves which might account for this conversion of NR into smaller CR species observed in crude extracts. However, they had been reported by other workers (Mikola and Kolehmainen, 1972) and leaf extracts from other plant species have been shown to contain proteinases potentially capable of degrading NR (Dalling *et al*, 1976; Ragster and Chrispeels, 1979; Discussion Section III).

Section II A MODEL FOR HIGHER PLANT NR

The exact relationship between the small CR species observed in crude extracts of barley leaves and after tryptic cleavage of purified NR to the NR complex is very important to understand in order to fully elucidate the structure of the enzyme.

We initially proposed a model for higher plant NR in which the CR species were envisaged to represent subunits and subunit associations (Wray *et al*, 1979). However, exhaustive genetic studies of the closely related NRs from *Aspergillus* (Cove, 1979) and *Neurospora* (Garrett and Amy, 1978) and less detailed, but comprehensive studies in higher plants (Mendel and Muller, 1979; Kleinhofs *et al*, 1980) have failed to provide evidence for more than one structural gene specifying the FAD and cyt b_{557}-containing subunit. As NR is clearly susceptible to proteolytic attack I would like, therefore to propose

that instead of representing intact subunits the CR species represent catalytically active, proteolytic fragments of NR. Since the same type of NADH-CR species are always found in extracts from older barley plants and after tryptic cleavage it follows that they must be produced by a specific mechanism and not just by random cleavage.

Proteolytic cleavage is now recognised not always to proceed according to an all or none process. The situation where only a few bonds are hydrolysed leading to more or less stable derivatives if often encountered (see Kirchner and Bisswanger, 1976). Cleavage is most unlikely to occur if a bond, however suitable, occurs in a rigid region of a polypeptide chain or buried inside a protein molecule. It is only in regions characterised by a high degree of local flexibility that peptide bonds will be able to take up a proper position in the active centre of the proteinase so that they can be hydrolysed (Drenth *et al*, 1968).

The catalytically active fragments released from the native enzyme on limited proteolysis are likely to represent domains of the enzyme. It is these functionally intact, independently folded regions of the polypeptide chain which are envisaged to be held together by flexible and loosely structured regions which are very sensitive to proteinase attack.

Early studies on the flavocytochrome b_2 from bakers' yeast suggested that the FAD and cytochrome components of the enzyme were contained in separate subunits (Mevel-Ninio, 1972). Subsequent work showed the enzyme to be polyglobular in structure (Naslin *et al*, 1973) with the FAD and cytochrome components located in separately folded regions of the polypeptide chain held together by regions hypersensitive to attack by proteinases (Jacq and Lederer, 1974). Tryptic cleavage of rat liver sulphite oxidase, a haemomolybdoprotein, results in the release of two major functional fragments, one of which contains the cyt *b* component while the other contains all of the molybdenum (Johnson and Rajagopalan, 1977; Southerland and Rajagopalan, 1978). It was concluded that the molybdenum and cytochrome components of the enzyme were contained in distinct domains which were covalently linked by an exposed hinge region of at least 30 amino acid residues.

It is highly probable, from such observations, that the FAD and cyt b_{557} components of higher plant NR are contained in separate functional domains while an additional domain may be involved in the binding of MoCo. As the CR partial activity of the NR complex is extremely heat labile, while $FMNH_2$-NR and MVH-NR activities are relatively stable (Wray and Filner, 1970) it seems likely that at least the FAD is contained in a separate domain. Such differential sensitivity is what one would expect if the CR activity was expressed in an independently

structured region of the NR complex.

This domain model for the subunits of NR suggests a possible relationship between NR and the smaller CR species apparently released from it (Fig.47). Proteinase attack at a hinge region (A) in either of the 100 000 MW subunits could lead to the release of the MW 40 000 (3.1S) CR species. Since FAD is required for CR activity (Garrett and Nason, 1969) this fragment probably contains at least the FAD domain along with one of the NADH binding sites of the native enzyme. The remaining MW 163 000 (6.8S) portion of NR would still be capable of expressing NADH-CR and NADH-NR activities due to the presence of one intact MW 100 000 subunit and MoCo. The two species possessing NR activity seen on isoelectric focusing of spinach NR (Notton *et al*, 1972) may be equivalent to the native enzyme and this nicked form. The MW 61 000 (3.1S) CR species could be generated from the MW 100 000 subunit by proteinase attack at a different hinge region (B) and may comprise the FAD domain (since the species possesses CR activity) and the haem domain, The presence of low levels of this MW 61 000 CR species suggests that this second proposed hinge region may be less exposed than that resulting in release of the 40 000 MW fragment. Alternatively, the 61 000 MW CR species may be rapidly broken down into the 40 000 MW CR species and a 20 000 MW protein fragment soon after it has been generated. The 5.6S CR species seen only in extracts from older tissue may represent the MW 100 000 subunit.

Fig.47

A Model for the Structure of Barley NR and for the Structure of the NADH-CR Species Postulated to be Derived from it

(1) The 100 000 MW monomer of NR.

I : the 40 000 MW FAD-containing domain

II : the 20 000 MW haem-containing domain

III : the 40 000 MW MoCo-binding domain

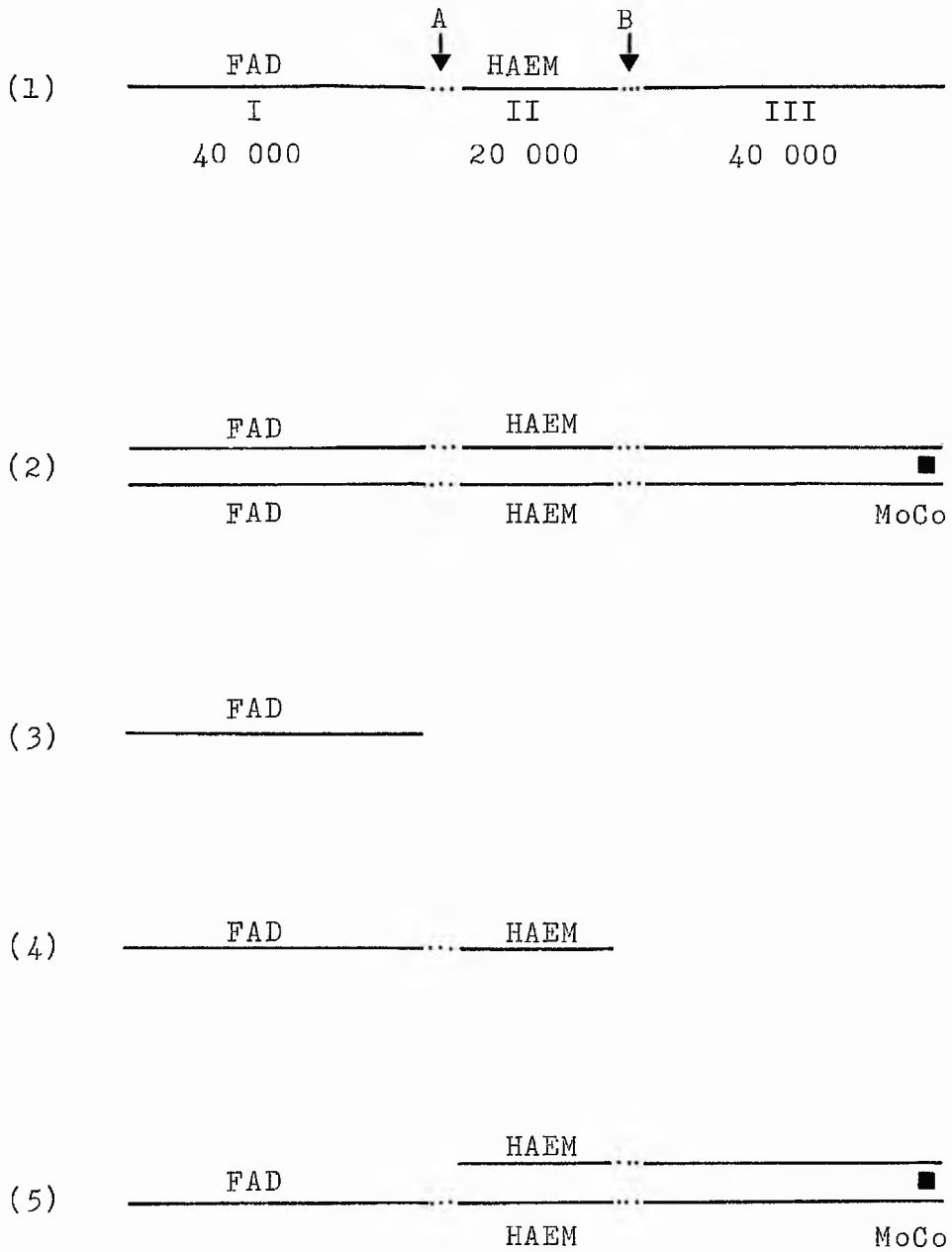
--- : denotes hinge regions.

(2) The 200 000 MW holoenzyme, comprising two 100 000 MW subunits plus one MoCo (■).

(3) The 40 000 MW CR species.

(4) The 61 000 MW CR species.

(5) The 163 000 MW CR species.


(1)
A
B
FAD
HAEM
I
II
III
40 000
20 000
40 000
(2)
FAD
HAEM
FAD
HAEM
MoCo
(3)
FAD
(4)
FAD
HAEM
(5)
HAEM
FAD
HAEM
MoCo

This model predicts the existence of an approximate 40 000 MW MoCo binding domain of the type observed to stabilise MoCo in *E. coli* NR which has a MW of approximately 40 000 (Amy and Rajagopalan, 1979).

The model also predicts that the 40 000 MW CR species does not contain a cytochrome component, thus suggesting that haem is not required for NADH-CR activity. Attempts to assess the haem content of this species proved unsuccessful (Chapter 4) however, its MW was confirmed using purified preparations. This species has subsequently been purified to electrophoretic homogeneity in this laboratory and assessed to contain no haem (J. McA. Campbell *et al*, 1984).

Very recently, Lê and Lederer (1983) have isolated a protease stable haem-binding domain from *N. crassa* NR made of a continuous stretch of peptide of approximately 100 residues (10-12 000 MW) which possess the native cyt b_5-like spectrum. This is a slightly smaller moiety than the approximate 20 000 MW predicted by this model for the haem binding domain of higher plant NR.

The model described here can be used to explain various observations reported in the literature:

CR species sedimenting in the 3-4S region after sucrose density gradient centrifugation of plant extracts are commonly observed but, the age-dependent production of the 6.8S and 5.6S CR species has not been reported in

any other system than that of barley (Small and Wray, 1980a; Chapter 2 of Results). However, closely similar species do appear to be present in spinach extracts under certain circumstances (Notton *et al*, 1976). In addition to the CR species of 8.1S and 3.6S normally seen in extracts of nitrate-grown plants, CR species of 6.9S and 5.5S were found in extracts from spinach plants which had been grown without Mo, with or without W but with ammonium as an additional nitrogen source to nitrate.

The observed characteristic of the NR seen in the *nit 3* mutant of *N. crassa* can also be explained in terms of the model. The *nit 3* mutant NR is smaller than the wild type NR and lacks NADPH-NR and NADPH-CR activities while retaining $FADH_2$ and MVH-NR activities (Antoine, 1974; Tachiki and Nason, 1983). It is feasible that this mutant NR lacks the FAD domain from each of its haemoflavoprotein subunits. This could be due either to a deletion or nonsense mutation in the part of the gene (exon, see Discussion, Section V) coding for the FAD domain or due to cleavage of an abnormal FAD domain from the subunit by proteolytic attack at a hinge region, either before or after assembly of the holoenzyme.

The model may also explain the characteristics of two NR deficient mutants of *Chlamydomonas reinhardii*, one uniquely containing NAD(P)H-diaphorase activity (mutant 104) and the other which only exhibits $FMNH_2$- or MVH-NR activities

(mutant 305). Reconstitution to give the whole NAD(P)H-NR complex has recently been achieved by mixing extracts of these two mutants (Fernandez and Cardenas, 1981). This was interpreted by the authors to indicate that *C. reinhardii* NAD(P)H-NR is a heteromultimeric complex consisting of two types of subunit separately responsible for the proximal and terminal activities of the enzyme. NR protein species equivalent to those possessed by the mutants could be produced from the 220 000 native complex of NR by trypsin treatment, namely a 45 000 species containing FAD and cyt b_{557} (Fernandez and Cardenas, 1983a; 1983b) and a 67 000 species containing Mo (Franco *et al*, 1984). Trypsin was envisaged by Franco *et al* (1984) to destroy the aggregation capabilities of the two subunits. However, an alternative explanation for these findings based on the domain model proposed here for higher plant NR is that trypsin cleaves the *C. reinhardii* approximately 110 000 MW subunit at a sensitive hinge region between the haem and MoCo binding domains (equivalent to hinge region B, Fig.47). It follows then that the NR protein species possessed by mutants 104 and 305 may arise from mutants in the parts of the genes coding for the MoCo binding domain and the FAD plus haem binding domains respectively.

The ability to reconstitute *C. reinhardii* NR from NR protein in the two mutants does not conflict with the suggestion that the protein species are domains or associations of domains rather than subunits. Cleavage

of the pyruvate dehydrogenase multienzyme complex of *E. coli* by trypsin gives rise to functional domains which can reassemble to produce a functional enzyme of very similar size to that of the native enzyme (Hale and Perham, 1979).

Likewise, the ability to reconstitute spinach NR from approximately 4S CR species and MoCo (Rucklidge *et al*, 1976) does not conflict with the domain model for NR since the 3-4S region of the gradient would be likely to contain both the 40 000 and 60 000 MW CR species plus the postulated approximately 40 000 MW MoCo binding domain.

SECTION III NR · INACTIVATORS

Two broad types of NR inactivator have been reported in the literature. One type associates with the NR complex in such a way as to cause a loss of NR activity which may or not be reversible. Such binding inhibitors have been identified in soybean leaves (Jolly and Tolbert, 1978) and rice cells (Yamaya and Ohira, 1976; 1977; 1978a; 1978b). Inhibitors of the type described from rice cells are unlikely to be responsible for the age-dependent, BSA-blockable inactivation of NR in barley leaves since the inactivating activity of the rice inhibitor was found to be unaffected by BSA and was most active in younger tissue.

The second mechanism by which NR may be inactivated is by proteolysis.

Wallace has described, in some detail, a NR-inactivating enzyme isolated from maize roots which, although specific for NR, is also capable of inactivating yeast tryptophan synthase (Wallace, 1978). Maize scutellum inorganic pyrophosphatase, glutamine synthetase and glutamate synthase are also inactivated by the enzyme but maize scutellum phosphofructokinase, nitrite reductase and glutamate dehydrogenase are relatively insensitive (Batt and Wallace, 1983). The enzyme is able to hydrolyse casein, is inhibited by PMSF and is considered to be a serine proteinase. Casein protects NR activity in crude extracts from inactivation therefore it is possible that it acts as an alternate substrate for the serine proteinase. The main site of action of the maize root proteinase is thought to be the NADH-CR component of NR, loss of NADH-NR activity occurring at a faster rate than that of $FADH_2$-NR or BVH-NR (Wallace, 1975b; Batt and Wallace, 1983). In addition, the 4S CR species thought to be related to NR is also inactivated preferentially by the proteinase (Wallace and Johnson, 1978; Smith, 1983). The effect of the enzyme on the $FADH_2$-NR component was shown to vary between species (Wallace, 1975b).

The maize root proteinase increases in activity with root age (Wallace and Shannon, 1981) to become the predominant protease species in that tissue. It is

interesting to note that despite this fact, PMSF which causes complete inhibition of the proteinase, only partially prevented loss of NR activity in crude extracts (Wallace, 1975a) implying that other proteinases are active against maize NR in crude extracts.

In the absence of an adequate supply of an homogeneous sample of higher plant NR, Yamaya *et al* (1980b) subjected *Chlorella* NR to the maize NR-inactivator and looked for structural changes in the enzyme by measuring mobility on non-denaturing polyacrylamide gels. The major protein band of *Chlorella* NR, which had MVH-NR activity, was shifted towards the anode after incubation with the maize protein and NADH-NR activity was detected in a different position from the MVH-NR activity thus demonstrating breakdown of NR and showing unequivocally that inactivation of NR by the maize NR-inactivator is by proteolysis.

Recently, Batt and Wallace (1983) performed similar limited proteolytic cleavage experiments on NR from maize scutellum and analysed the products by sucrose density gradient analysis. After incubation of the NR sample with maize root proteinase a major loss of both 7.2S NR-CR and 3.1S CR activity was observed. Very similar results were obtained after incubation of purified barley leaf NR with maize NR-inactivating factor II described in this thesis (Chapter 6, Fig.35). In contrast Batt and Wallace (1983) found that after trypsin treatment of the maize enzyme there was loss of 7.2S NR-CR but an increase of the 3.1S CR species. Such observations on the effect of

trypsin on maize scutellum NR are strikingly similar to those made here on the effect of trypsin on barley leaf NR (Chapter 5, Fig.32), suggesting that the maize and barley enzymes have structural features in common.

Batt and Wallace (1983) commented that if the small CR species had the same assay properties and is all released from the NR complex then one would have expected the increase in the 3.1S CR to exceed loss of NR-CR activity. However, in both systems, although the ratio of loss of NR-CR to increase in 3.1S CR was found to fluctuate in different experiments, increase in the activity of the 3.1S CR species never exceeded loss of 7.2 or 7.7S CR activity.

The fact that 3.1S CR species were present in untreated control preparations of maize scutellum NR indicates that there must be a mechanism whereby this species is generated *in vitro*. This is not likely to involve the maize proteinase since it has been shown to preferentially attack the dehydrogenase portion of the NR complex and the 3.1S CR species (Wallace and Johnson, 1978).

A heat-labile factor which has a negative effect on the stability of NR has been identified in wheat leaf extract (Sherrard *et al*, 1979b). The factor (MW 37 500) had no effect on highly purified wheat leaf NR or ribulose 1,5-bisphosphate carboxylase. The inactivating factor was more active towards NADH-NR activity than

either $FMNH_2$-NR or MVH-NR and the NADH-ferrocyanide reductase activity was the least sensitive. No conclusive evidence was obtained by the authors to indicate that inactivation by the factor was due to proteolysis since it had no detectable hydrolytic activity towards casein, haemoglobin or several artificial substrates. It was however inhibited by TLCK and α N-benzoyl-L arginine which are specific inhibitors of trypsin-like enzymes. The factor was not inhibited by PMSF and had a pH optimum of 7.0.

As the wheat NR inactivating factor is least effective towards the diaphorase activity of NR it is possible that, like trypsin, it generates catalytically active fragments of NR but since the products of inactivation were not analysed by Sherrard *et al* (1979b) this awaits confirmation.

Barley NR has been shown to be susceptible to proteolytic cleavage by trypsin (Chapter 5). However, if limited proteolysis of NR occurs in crude extracts of barley leaves it is clear that it is due to a proteinase which is not totally inhibited by PMSF or 1,10-phenanthroline since these proteinase inhibitors only slightly stabilise NR (Chapter 2).

Two acid endoproteinases have been identified in barley primary leaves, subsequent to the completion of the work described in this thesis, by Miller and Huffaker (1980a; 1980b). One, a serine proteinase, is inhibited

by PMSF whilst the other, a thiol endoproteinase is inhibited by leupeptin. Leupeptin was found by Wray and Kirk (1981) to be extremely effective in stabilising NR activity in extracts of barley *cv* Golden Promise primary leaves and preventing conversion of NR to the 3.1S and 3.8S NADH-CR species. Such results suggest that the major proteinase active against NR in barley leaf extracts is the thiol-dependent acid endoproteinase described by Miller and Huffaker (1980a; 1980b). This thiol endoproteinase constitutes around 80% of the total endoproteinase activity in barley leaf extracts and thus accounts for PMSF being ineffective in stabilising NR.

Miller and Huffaker (1981) reported that BSA could not prevent hydrolysis of ribulose 1,5 bis carboxylase by the two barley leaf proteinase. It was also shown that BSA, at concentrations (mg/ml) as much as 2-fold greater than that of the enzyme, proved a poor substrate for the proteinases. This suggests that the protective effect of BSA toward NR activity in crude extracts of barley is not due to its ability to act as an alternative substrate for the leupeptin-sensitive proteinase.

The thiol-dependent proteinase was found to have a pH optimum of 5.7 (Miller and Huffaker, 1980b) which is consistent with the fact that alkaline pH is a major stabilising property of the novel buffer described by Kuo (Kuo, 1979; Kuo *et al*, 1980) and phosphate extraction

buffers described here (Chapter 2, Fig.19). In addition,the proteinase was found to be extremely dependent on thiols for stability. Exogenous thiols such as cysteine and DTT also stabilise NR by preventing disulphide bond formation and are almost exclusively included in extraction buffers for NR, thus also leading to stabilising conditions for the endoproteinase.

Attempts were made in this laboratory to identify and isolate the proteinase(s) responsible for degradation of barley leaf NR *in vitro* (Smith, 1983). One NR-inactivating factor was partially purified from 10 day old barley leaves which was inhibited by EDTA and 1,10-phenanthroline but not by casein or BSA. Like the thiol endoproteinase described above it was completely unaffected by PMSF however,it was only slightly inhibited by leupeptin. As cysteine was not included in the buffers employed by Smith (1983) in the purification procedure for the NR inactivating factor it is unlikely that it represents the thiol endoproteinase described by Miller and Huffaker (1980a; 1980b;1981; 1982). In addition sucrose density gradient analysis of purified barley NR incubated with the factor isolated by Smith (1983) did not produce evidence for the formation of smaller enzymatically active species during the inactivation process (Chapter 6, Fig.36). The factor is therefore unlikely to be responsible for the phenomenon observed in crude extracts of barley leaves, unless of course the partially purified NR inactivator preparation also contained substances capable of inactivating the small CR species.

A leupeptin sensitive endoproteinase has recently been purified from *Hordeum distichium* leaves by Hamano *et al* (1983; 1984). The endoproteinase was found to have a pI of 4.05 and an optimum pH of 6.0 which are similar to those reported for the thiol proteinase isolated from senescing leaves of *Hordeum vulgare* by Miller and Huffaker, (1980a; 1980b). However, pH stability of the enzymes and K_m values for azocasein differed therefore it was concluded that the enzymes were not identical. The products of NR degradation were not analysed by these authors, however they did show that although the purified thiol proteinase inactivated $FMNH_2$-NR and MVH-NR as well as the NADH-NR activity, it had only a slight effect on the NADH-CR activity. This suggests that, like trypsin, the barley thiol endoproteinase degrades NR into CR active fragments.

SECTION IV EFFECT OF INSTABILITY OF NR *IN VITRO* ON THE OBSERVED STRUCTURE OF THE PURIFIED ENZYME

J. McA. Campbell and Wray (1983), working in our laboratory, have recently investigated the effect of various purification conditions on the observed characteristics of the purified enzyme. The conditions ranged from one where no attempt was made to protect the enzyme from proteolysis to one which was likely to afford a high level of protection.

NR purified by these authors according to the method described in this thesis (Chapter 3), in which no attempt

was made to minimise proteolysis, resulted in an enzyme preparation of rather low specific activity (0.27 units) which migrated on native gels of different percentage polyacrylamide as a single protein with a MW of 205 000. When run on denaturing gels 2 major protein species of MW 59 000 and 38 000 were observed which accounted for over 90% of the dye binding, together with a faint band at 103 000 MW which presumably represented the monomer of NR. A major band of 60 000 MW was also observed on SDS gel electrophoresis during the work described in this thesis (Chapter 3). When NR was purified using Kuo's buffer containing 10μM leupeptin, conditions under which proteolysis of NR might have been expected to be minimal, a 25-fold increase in the specific activity of the enzyme preparation resulted. The NR migrated as a single band at 205 000 MW in non-denaturing gels but SDS gel electrophoresis showed a quite different pattern of bands to that previously observed. The major protein band corresponded to 103 000 MW and while bands corresponding to lower MW protein species were still present, they were very much reduced in intensity.

Thus, NR purified under conditions where proteolysis is not blocked has a low specific activity, perhaps due to a high degree of nicking of the subunits, and dissociates on SDS gels to reveal the presence of only small amounts of intact subunits. When attempts were made to reduce proteolysis, fewer subunits were nicked, as judged by the greater proportion of the 103 000 MW band on SDS gels and the higher specific activity of the enzyme. It would

appear that even highly nicked molecules behave on non-denaturing gels as though subunit integrity had been retained. Proteolytic nicking resulting in a decrease in specific activity has been observed for yeast pyruvate dehydrogenase complex (Kresze and Ronft, 1981) and yeast tryptophan synthase (Bartholomes *et al*, 1979).

Proteolytic nicking of NR as described for barley cv Golden Promise NR may explain the complex patterns seen after SDS gel electrophoresis of purified spinach NR (Notton and Hewitt, 1979) and *N. tabacum* NR (Mendel and Muller, 1980). Recently W.H. Campbell (1982) also reported that the number of protein staining bands observed after SDS gel electrophoresis of purified squash NR was dependent on the purification procedure, either resulting in bands corresponding to MW 44 000 and 75 000 or 110 000 alone. He concluded that the 110 000 MW species was likely to be an adduct of the two smaller polypeptides but a labile attachment between the two was not ruled out. I consider it highly likely that this labile attachment proposed by W.H. Campbell is in fact an inter-domain hinge region.

It has not yet been shown by J. McA. Campbell and Wray (1983) that the 59 000 and 38 000 protein bands seen by them on SDS gel electrophoresis are equivalent to the 61 000 MW (3.8S) and 40 000 MW (3.1S) CR species seen in cell-free barley leaf extracts, but equivalence is considered likely. However, why subunit integrity is maintained on non-denaturing gel electrophoresis, but not

on sucrose density gradient centrifugation or gel filtration (Small, 1980) is not known.

The suspected proteolytic nicking of NRs from sources other than higher plants, as judged by heterogeneous bands on SDS gels, may not be the result of cleavage at hinge regions to release domains as often the difference in calculated MWs is probably too small. For example, estimates of the subunit MW of *N. crassa* NR (115 000 and 130 000) (Pan and Nason, 1978) and *Rhodotorula glutinis* (115 000 and 118 000) (Guerrero and Gutierrez, 1977) are likely to come into this category. *Chlorella* NR may also be susceptible to this type of proteolytic nicking since microheterogeneity was observed on C terminal analysis of the enzyme by Solomonson (personal communication).

NR has recently been purified by Kuo *et al* (1982b) from the barley cultivar Stepoe. The purified enzyme preparation was found to contain additional, abundant protein species of MW 60 000 and 41 000, amongst others, as judged by electrophoresis under non-denaturing conditions. Second dimensional SDS gel electrophoresis revealed a major band at MW 110 000 (NR monomer) together with these additional species. Peptide mapping of the MW 60 000 and 41 000 species with *S. aureus* protease V8 and endoproteinase lys-C was interpreted to show no homology with the MW 110 000 subunit. It was therefore concluded that the 60 000 and 40 000 MW polypeptides could not represent breakdown products of the 110 000 NR subunit but rather represented contaminants of the affinity purified NR preparation.

It is possible that the different non-denaturing electrophoresis conditions employed by Kuo *et al* (1982b) and J.McA. Campbell and Wray (1983) may have resulted in the species comigrating under one set of conditions while not in the other. When *Chlorella* NR was run on 6% polyacrylamide gels with tris barbital buffer there was only one protein band while with a 7.5% polyacrylamide gel and Tris glycine buffer system the purified NR had one major and two minor protein bands (Yamaya *et al*, 1980a). However, as the Golden Promise NR was shown to be electrophoretically homogenous in different percentage non-denaturing gels this is considered unlikely (J.McA. Campbell and Wray, 1983).

J.McA. Campbell and Wray (1983) commented that the protein species of MW 60 000 and 41 000 observed by Kuo *et al* (1982b) would have been excluded by the Biogel A 1.5 m gel filtration step employed by them but which was not included in the purification scheme described for Steptoe NR. They therefore concluded that the MW 60 000 and 41 000 protein species seen by Kuo *et al* (1982b) as pronounced protein contaminants after non-denaturing gel electrophoresis are unlikely to be the same as the MW 59 000 and 38 000 protein species seen by them only after SDS gel electrophoresis.

The nature of the CR species in *Hordeum vulgare* or Steptoe wild type and *nar* mutant extracts has recently been investigated by Narayanan *et al* (1983). Two NADH-CR species sedimenting at 8S and 4S were observed on sucrose density gradient analysis. The subunit MW of both the

8S (intact NR complex) and the 4S CR species was determined to be 110 000 by a combination of SDS gel electrophoresis and immunolocalisation. It was thus concluded that the 4S CR species represents the monomeric form of NR and not a degradation product of NR. The authors commented that proteolytic degradation products of NR were unlikely to be present in the 4S region of the sucrose gradient since none were detected by the anti-NR immunolocalisation although the anti-NR serum completely inhibited the NADH-CR activity in the 4S region.

The amount of the 4S CR species present in *nar* 1d and *nar* 2a mutant extracts as a proportion of the total CR species was shown by Narayanan *et al* (unpublished observations) to be dependent on the *in vitro* conditions. One may speculate that if immunolocalisation and MW determination of the species sedimenting in the 4S region were to be carried out under conditions which afforded no protection against proteolysis, then heterogeneity of NR protein associated with the 4S peak may then be observed.

Since CR species of 4S are present in extracts of barley cv Steptoe even under the stabilising conditions described by Narayanan *et al* (1983) this would suggest that dissociation of NR subunits is not prevented under these conditions, which have been devised to minimise proteolysis. It is therefore likely that dissociation into monomers of NR occurs in extracts of barley cv Golden Promise in addition to degradation of the monomers

to contribute to the instability of NR *in vitro*.

J.McA. Campbell and Wray (1983) showed that even using Kuo's buffer with leupeptin, the enzyme still appeared to exist in the nicked form, although to a much lesser extent than when no precautions were taken against proteolysis. At first sight, it would appear contradictory that Kleinhofs' group never detect any NR related protein species less than 110 000 MW. However, while proteolytic nicking of the NR into functional domains would appear to occur under the growth, extraction and purification conditions described here (Chapter 3) and by J.McA. Campbell and Wray (1983) for the Golden Promise cultivar it does not necessarily follow that it occurs during all growth, extraction and purification procedures.

Seedling growth temperature is known to affect NR activity in plants. A growth temperature of 16°C was found to yield the most stable NR activity on extraction of barley leaves by Kuo *et al* (1982a). The decreased stability of the Golden Promise NR may therefore be contributed to by the growth temperature of 26°C employed in our laboratory. Preliminary experiments (J.McA. Campbell, personal communication) with the purification of NR from barley or Golden Promise grown at 16°C, revealed multiple bands on electrophoresis of the purified enzyme in non-denaturing gels (despite the inclusion of a gel filtration step). Whether these bands are equivalent to the bands observed by Kuo *et al* (1982b) as contaminants of purified preparations of Steptoe NR remains to be seen.

It was also found during the investigations described in this thesis that seed age and storage conditions affected stability of leaf NR. Old seed which had been stored at 26°C in the light gave rise to leaf tissue which by the 6th day contained highly labile NR. Fresh seed stored below 20°C in the dark, however, was required to be grown to day 10 before NR in leaf tissue became labile.

The form in which nitrate is administered to the plant has also been shown to affect the amount of NR in leaf tissue. Schrader *et al* (1972) have reported that the levels of NR activity were higher in corn plants supplied with a combination of both ammonium and nitrate as compared to nitrate alone. Feeding with mixtures of nitrate and ammonium in different ratios can also affect growth rate and yield : increases of up to 50% in both resulting from controlled ammonium addition to nitrate supply were reported by Reisenauer (1978). Lewis *et al* (1982) recently investigated this phenomenon in barley and found that NR activity was less in leaves when the plants were supplied with ammonium and nitrate. In contrast J.McA. Campbell and Wray (1983) obtained a 3-fold increase in the NR activity extracted from 4 day old Golden Promise barley shoots by growing plants with half Hoagland nutrient solution containing 10mM KNO_3 and 30mM NH_4Cl rather than 15mM KNO_3 alone. NR purified in the same way as described in this thesis but from plants grown with nitrate and ammonium gave a 3 fold increase in specific

activity implying that nutrition also plays a role in the stability of NR. It is not known how this is effected but Sihag *et al* (1979) proposed that the two-fold increase in NR activity on addition of ammonium along with nitrate to dark grown peas may be dependent on regulatory protein(s) synthesised in response to ammonium. It is significant to note that Kuo *et al* (1980) employ a nutrient solution which contains an ammonium salt.

The option remains to be considered that the NRs in barley cultivars Steptoe and Golden Promise are genetically different. There has been much interest in NR in relation to its use as a possible selection criteria in plant breeding programmes and genotypic differences in NR levels have been reported in maize (Beevers and Hageman, 1969; Hageman *et al*, 1976; Deckard *et al*, 1973), wheat (Hageman *et al*, 1976; Dalling and Loyn, 1977; Austin *et al*, 1978) and barley (Eck and Hageman, 1974; Bowerman and Goodman, 1971).

Early attempts to correlate NR activity with various economic parameters did not take into account the fact that NR is unstable *in vitro*. However, the ranking of various cultivars of wheat with respect to NR activity did not markedly alter when casein was used for extraction (Sherrard and Dalling, 1978), inferring that variability in NR activity in the various cultivars does have a genetic basis and is not merely an artifact of extraction. Genetic differences in NR activity may not be due wholly

to differences in the enzyme *per se* as genetic differences in NR inactivators may also be important. Evidence for this comes from the observation that although the presence of casein increased extractable NR in some cultivars of wheat, it afforded no improvement in others (Sherrard and Dalling, 1978). The discovery and investigation of high NR - low protease and low NR - high protease genotypes of maize (Reed *et al*, 1980) are likely to prove most enlightening with regard to this subject.

SECTION V EVOLUTION OF THE ASSIMILATORY NRs

Although all assimilatory NRs are capable of catalysing the same types of reactions and all contain the same prosthetic groups, current data indicate that the specific structure of NR is species dependent. Although some of the differences observed between estimated MW of native enzymes and stochiometry of subunits may be accounted for by the susceptibility of the enzyme complex to proteolysis, it is clear that the algal enzymes are much larger than those from higher plant and fungal sources. The differences observed in structural features are also apparent in other properties of NR, for instance in their kinetic mechanisms. Once again, proteolytic modification of NR has been suggested to account for some of the differences observed (Renosto *et al*, 1982).

Chemical similarity and structural homology of NRs from different sources has been investigated by use of immunological cross reactions (Smarrelli and Campbell, 1981; Funkhouser and Ramadoss, 1980). Such investigations are considered valid since it has been reported that the majority of evolutionary substitutions in proteins are immunologically detectable (White *et al*, 1978). Such studies have also been used to examine evolutionary divergency of proteins such as lysozyme, catalase, trypsin and cytochrome *c* (Arnon, 1973).

Smarrelli and Campbell (1981) used antiserum to squash NR to compare immunological cross reactivity of NR from spinach, corn, soybean, *N. crassa* and *C. vulgaris*. All forms of NR had antigenic sites in common with squash. Spinach NR was found to be the most closely related to squash NR followed by the other higher plant enzymes, *Chlorella* then *Neurospora*. Funkhouser and Ramadoss (1980) have also shown that *C. vulgaris* NR had common antigenic determinants with spinach and *N. crassa*.

Recently Kuo *et al* (1982b) analysed the amino acid composition of barley NR subunit and found its composition to closely resemble that reported for *Chlorella* (Giri and Ramadoss, 1979) and *Ankistrodesmus* (De La Rosa *et al*, 1981). Thus a high degree of homology exists among the eukaryotic NRs.

It is well established that NR from eukaryotic sources contains a MoCo which is common to other

molybdoenzymes including rat liver sulphite oxidase (Johnson *et al*, 1980). Recently, evidence has been reported to suggest that the haem binding domain of a variety of enzymes may have a common ancestral origin (Le and Lederer, 1983).

A haem binding domain (MW 10 - 12 500) was isolated after selective proteolysis of native *N. crassa* NR. A similar procedure has been used for isolating the haem binding domain from flavocytochrome b_2 (Labeyrie *et al*, 1966; Gervais and Tegoni, 1980) and sulphite oxidase (Ito, 1971; Guiard and Lederer, 1977; Johnson and Rajagopalan, 1977). Since the cytochrome components of all three enzymes have been shown to possess identical absorption maxima it has been suggested that they are likely to represent very similar, if not identical structural components. The purification of haem domains has therefore afforded the opportunity to assess this hypothesis.

Sequence results of the *N. crassa* haem-binding domain showed an unmistakable similarity with other proteins of the cyt *b* family. Interestingly, the results of Le and Lederer (1983) indicated the similarity to be highest with microsomal cyt b_5.

It has been suggested that the degree of sequence similarity is indicative of a divergent evolutionary relationship between the haem-binding domains of assimilatory NR and those of cyt b_5, flavocytochrome b_2 and sulphite oxidase.

Thus we have a situation in which all assimilatory NRs are likely to share a common ancestor but in addition the MoCos and haem binding domains share a common evolutionary relationship with components of other enzymes. It seems likely, therefore, that the FAD binding domain will also be found to be related to the FAD domains from other flavoproteins. The 3.1S CR species isolated as described in this thesis (Chapter 4) may help to solve this issue in the future.

NR, along with the other redox enzymes described here, are multifunctional proteins, the subunits consisting of a single polypeptide chain but having multiple catalytic or binding functions organised into domains. It has been postulated that multifunctional proteins may have evolved from smaller, globular proteins. Some evidence for this comes from the fact that some enzyme systems that catalyse sequential reactions in a metabolic pathway occur in different organisms as individual proteins, multienzyme complexes or multifunctional enzymes (Calvo and Fink, 1971; Crawford, 1975; Truffa-Bachi and Cohen, 1973; Zalkin, 1973). It has therefore been postulated that evolution of multifunctional enzymes has occurred from small proteins via multi-enzyme complexes (Smith, 1970). Some of the processes envisaged to be involved with this process have been demonstrated experimentally: partial or full gene duplication and subsequent gene rearrangements by gene translation and gene fusion.

It has recently been discovered (see Crick, 1979) that many, and probably most, genes from eukaryotes occur in fragments spread out along DNA. In between the gene fragments (exons) are long intervening stretches of DNA (introns) whose functions are unknown. Transcription of the complete gene (introns and exons) results in the production of heterogeneous nuclear RNA. The non-coding sections of genetic material are then spliced out of the RNA in order to produce true messenger RNA. One explanation for the existence of introns is that they represent itinerant stretches of DNA and that the splicing system has arisen in order to protect the integrity of the gene during expression. However, it has been considered more likely that it is the gene segments or 'minigenes' that are the mobile entities (Lewin, 1979). Thus a 'minigene' which codes for a length of polypeptide which itself constitutes a functional area or domain, could readily be combined with another 'minigene' to form a completely new protein. This is obviously an advantage to evolution as new genes could be formed from pre-fabricated gene sections.

If this theory turns out to be a general phenomenon then it is easy to envisage how multifunctional proteins like NR, sulphite oxidase and flavocytochrome b_2 may have evolved.

It has been noted by Kirchner and Bisswanger (1976) in a review on multifunctional proteins that such proteins

are often discovered in a haphazard way as nicking of multifunctional enzymes can generate apparently non-interacting enzymes or a multienzyme complex without drastic changes in the properties of the enzymic activities. Higher plant NR would appear to illustrate this well.

The susceptibility of the enzyme to proteolytic nicking has obviously caused problems in the purification and characterisation of the enzyme (see Introduction). However, this susceptibility to nicking has been exploited to obtain more information about the quaternary structure of the enzyme and the evolutionary origins of NR. Whether or not limited proteolysis of NR has a role to play *in vivo* will be investigated in the following section.

SECTION VI ROLE OF PROTEOLYSIS IN REGULATION OF NR ACTIVITY *IN VIVO*

The amount of any individual protein in a plant cell is a function of its turnover, that is the rate of synthesis and the rate of degradation of that protein. Although the rate of turnover of a protein may vary with the age of the plant cell and may differ between classes of proteins it is considered highly likely that all plant proteins are subjected to continual turnover. Tobacco cell NR has been shown by Zielke and Filner (1971) to turnover whether in a steady state, induction or decay phase, indicating the involvement of a constitutive proteolytic mechanism. The appearance of low levels of

the 40 000 and 61 000 CR species in young barley leaf extracts in which NR activity is stable (Chapter 2, Fig.11a) and their presence following extraction with buffer containing 3% BSA (Chapter 2, Fig.13b) suggests that proteolytic breakdown of NR occurs due to normal turnover of the enzyme. Thus, the levels of these species present in extracts from young plants or in extracts from older plants prepared in the presence of 3% BSA may reflect the extent of *in vivo* turnover of NR.

It is important to differentiate between the involvement of proteinases in normal turnover and their role during senescence. Presumably, during senescence proteins could be degraded indiscriminately while at other times, some sensitive control must be achieved. The observation that proteolytic enzymes capable of degrading NR are present throughout the entire life-span of the maize root (Wallace and Shannon, 1981) coupled with the finding of a distinct diurnal rhythm in levels of extractable activity of NR in wheat leaves (Sherrard *et al*, 1979a; see also later) suggest that proteinases are associated with normal protein turnover and are not solely the components of senescing tissues. If this is so, then clearly the rate at which these proteinases degrade NR in the non-senescing cell must be controlled.

Regulation of NR degradation could depend on either the protein (substrate) to be degraded, the proteinase(s) or depend on a combination of the two.

Studies with mammalian (Dice and Goldberg, 1976; Schimke, 1975) and plant (Gustafson and Ryan, 1976) proteins indicate that their susceptibility to degradation is influenced by various physical properties of the protein. These workers have shown that larger proteins have higher turnover rates. Higher plant NR is a large protein and this correlates well with the short half lives of 1½ - 6 h which have been measured for the enzyme in several plants (Oaks *et al*, 1972; Notton *et al*, 1972; Radin, 1974; Aslam and Oaks, 1976). It has also been shown that acidic proteins undergo more rapid rates of catabolism than neutral or basic ones. Spinach NR is reported to be an acidic enzyme with an isoelectric point at pH 5.0 (Notton and Hewitt, 1979) and amino acid analysis of barley NR has shown a predominance of acid residues Kuo *et al*, 1982b).

In addition to structural characteristics, susceptibility to degradation may be influenced by changes in the conformation of the protein. Perhaps a change in the conformation of NR may result in exposure of proteinase-sensitive hinge region(s) and thus act as a trigger for degradation (Schimke, 1975).

Absence of required cofactors and substrates has been shown to influence *in vivo* stability of many proteins. NR from molybdenum-deficient spinach plants has been shown to be turned over more rapidly than from molybdenum-sufficient plants (Notton *et al*, 1976). As previously noted in this discussion (Section II),

analagous if not identical NADH-CR species are present in extracts from older barley plants (Chapter 2, Fig.11b; Small, 1980) as are found in extracts from molybdenum-deficient spinach plants (Notton *et al*, 1976). As these species are not observed in molybdenum-sufficient spinach plants of the same age (Notton *et al*, 1976) it may be concluded that these CR species are likely to be generated *in vivo* as a consequence of molybdenum deficiency. Thus, in the absence of molybdenum the altered NR protein appears to be broken down and indeed enhanced turnover of altered proteins is well documented (Platt *et al*, 1970; Lin and Zabin, 1972; De Simone *et al*, 1974).

Changes in conformation of NR may also confer stability on the enzyme perhaps by burying a proteinase sensitive hinge region. When *Chlorella* NR is reduced (that is reversibly inhibited) it has been found to be resistant to trypsin (Howard and Solomonson, 1978). NR from wheat leaves has been shown by Street and Feller (1982) to be protected from inactivation by an extract from senescing leaves when NADH was present while addition of NAD^+ or of adenylates had no effect. In contrast, glutamine synthetase from wheat leaves was protected by ATP but not by pyridine nucleotides. The action of trypsin or maize root proteinase on maize scutellum NR was, however, not altered by the presence of nitrate, FAD or cysteine (Batt and Wallace, 1983).

Conformational changes of NR may also be brought about in response to variations in ionic strength and

pH or to the interaction of other molecules, not only substrates and cofactors, with the enzyme. These conditions could arise as a result of normal metabolic activity, aging or some form of environmental stress.

A NR-stabilising factor has been identified and partially purified from cotton cotyledons by Purvis *et al* (1980). The authors favoured the suggestion that the factor protected NR from physical denaturation by binding to the enzyme. Since this 'factor' was only partially purified it may merely stabilise NR by increasing the protein concentration of the extract, as has been suggested as the mode of action of other exogenous proteins such as BSA and casein. However, the 'factor' was effective at a much lower concentration than BSA or casein therefore the effect of this plant protein preparation may not be entirely non-specific.

Sherrard *et al* (1979a) have isolated two types of factor, one (II) negative and two (I and III) positive, which affect the *in vitro* stability of NR from wheat leaves. The levels of these factors were shown to be subject to diurnal variation. Factors I and III were highest when *in vitro* NR activity and stability were highest while Factor II was apparently out of phase, its maximum level coinciding with the time of minimum *in vitro* NR activity and stability. Data obtained by these authors favoured the direct interaction of Factors I and III with NR resulting in simultaneous protection against denaturation and proteolysis.

An alternative means of regulating the rate of degradation of protein *in vivo* is by regulating the activity of the degrading system and this may be brought about by *de novo* synthesis, activation-inhibition or compartmentalisation of the proteinase.

In some situations where light is required for induction and maintainance of NR levels, loss of activity in the dark can be retarded by cycloheximide. This has been interpreted as evidence for a degradative system whose synthesis is blocked by cycloheximide (Travis *et al*, 1969).

Many proteinases have specific inhibitors which serve to prevent unwanted proteolysis (Laskowski and Kato, 1980). Several authors have reported the existence of factors which bring about loss of NR activity. However, until they have been purified and tested with purified preparations of NR-inactivating factors it is not possible to determine whether they act by inhibiting the inactivators or by directly protecting NR from inactivation.

In plant cells, control of protein degradation by compartmentalisation is not well documented but the existence of plant lysosomes, in the form of the plant vacuole, has been postulated (Parish, 1975a, 1975b). However, it is difficult to comprehend how lysosomes could be involved in protein degradation in the steady-state situation in which degradative rates differ among proteins unless of mechanism for discriminating between the proteins exists. Alternatively it has been suggested that there

are specific proteinases for specific proteins or classes of proteins.

It is not known whether the fact that NR is susceptible to limited proteolysis is relevant to this however, the discovery of group-specific proteinases, such as those active against the apo- form of certain pyridoxal phosphate-dependent enzymes (Katanuma *et al*, 1976) has led to the suggestion that at least some enzymes may be degraded via a sequential process. Degradation may occur via a limited proteolytic attack by a relatively specific proteinase followed by further attack by other, more non-specific proteinases, either in the cytoplasm or in the vacuole (Segal, 1976).

Caution must be observed when attempting to interpret data from the *in vitro* hydrolysis of protein substrates by so-called specific proteinases. An endoproteinase that hydrolyses a protein substrate *in vitro* may not do so *in vivo* because of a differential compartmentation within the cell. It is therefore important to determine the subcellular location of endoproteinases. Very recently it has been discovered that the leupeptin-sensitive endoproteinase described by Miller and Huffaker (1980a, 1980b) from barley leaves is located in the vacuole (Thayer and Huffaker, 1984). Although the intracellular distributions of the barley leaf endoproteinase described by Hamano *et al* (1983; 1984) and the maize root proteinase described by Wallace (1975b) are unknown it is likely that they too have vacuolar (lysosomal) locations. As NR

is located in the cytosol of the plant cell and normal protein turnover in animal and plant cells is considered to involve a non-lysosomal route (Ballard, 1977) it is possible that these so-called specific endoproteinases may not have a role in the degradation of NR *in vivo*. There may, however, exist endoproteinases in the cell that hydrolyse NR but which are not detected by standard proteolytic assays.

A combination of mutant technology and biochemistry should enable us to find out which processes are regulated by proteolysis within the cell and which proteinase catalyses which intracellular process. Experiments directed towards this goal in yeasts have lead to isolation of mutants lacking two endoproteinases (Mechler and Wolf, 1981). However, although *in vitro* studies suggested these endoproteinases to be involved in specific events *in vivo*, their absence from mutants did not seem to affect these processes *in vivo*. The mutants lacking these proteinases did, however, degrade much less intracellular protein and thus it was suggested that their role *in vivo* must be of an unspecific nature, like, for example, protein degradation under conditions of nitrogen starvation. It was therefore concluded by Wolf (1982) that unknown proteinases responsible for more specific proteolysis must exist in *S. cerevisiae*. Since the leupeptin-sensitive endoproteinase in barley comprises 80% of the total proteolytic active species in barley leaves it is possible that it also has an unspecific role in regulating NR *in vivo*. Chromogenic peptide substrates

designed for the relatively specific serum proteinases have been employed in the search for new proteinases in yeasts (Achstetter *et al*, 1981) and many new proteolytic activities have been detected. It may therefore be possible to employ such substrates to assist in determining the *in vivo* relationship between proteinase activity and proteinase function and selectivity in higher plants.

Since higher plant proteinase mutants are not yet available and detailed investigations with homogenous samples of NR substrates and NR inactivators have as yet been limited, many studies on the regulation of NR have relied on the measurement of NR activity in various situations. Many of the studies, including the density labelling studies of Zielke and Filner (1971), do not allow a distinction to be drawn between degradation and inactivation of enzyme molecules (Gore and Wray, 1978). Similarly an increase in NR activity may not necessarily be due to an increase in enzyme synthesis as an increase in activity may be brought about by a reduced rate of enzyme degradation. In order to differentiate between *de novo* synthesis/degradation and reversible activation/inactivation of NR it is necessary to measure the amount of NR protein present regardless of enzyme activity and immunological techniques have been increasingly employed for this purpose.

Somers *et al* (1983) have shown, by rocket immuno-electrophoresis and SDS western blotting of barley shoot

extracts, that NR protein is not present unless the plant has received nitrate. As nitrate-dependent and light mediated increases in NR-CRM were similar to increases in NR activity, this suggested to them that NR was synthesised *de novo* in response to nitrate and light. In a similar study described in this thesis (Chapter 7), but employing a protection of inhibition assay for detecting NR-CRM, there was an indication that on induction of previously nitrate-less barley plants, NR-CRM measured in leaf extracts increased more rapidly than did NR activity. This observation is supported by the work of Smarrelli and Campbell (1982) who monitored the induction of squash NR using an enzyme-linked-immunoabsorbant assay. They showed rather complex changes in the antigenic material of crude extracts during induction and concluded that highly antigenic precursors of NR were first synthesised and then reorganised into active NR.

The presence of NR-CRM in ammonium-grown leaf extracts of barley was not investigated by Somers *et al* (1983) but Funkhouser and Ramadoss (1980b) showed that ammonium-grown *Chlorella* cells, which contain little active enzyme, contain much NR-CRM. A substantial amount of NR-CRM was always observed (Chapter 7) in extracts of barley plants grown in the presence of ammonium. Some NR activity, however, was always detected in such extracts. This phenomenon has also been noted in barley grown under sterile conditions under which one can discount inadvertant nitrate application (J. Ip, personal communication).

The method described in this thesis for estimating NR protein was not sensitive enough to determine whether induction of NR activity after administration of nitrate to ammonium-grown plants was due to activation of the high level of NR-CRM always observed in extracts of these ammonium-grown plants. Immunological methods of the type employed by Somers *et al* (1983) and Smarrelli and Campbell (1982) will be required before this issue can be resolved in higher plants.

Funkhouser and Ramadoss (1980) proposed that the formation of active NR in *Chlorella* cells occurs in two steps: amino acids are first combined to form an inactive protein product then the inactive precursor is converted into active enzyme in a process which apparently depends on another protein. It was concluded that the second step was suppressed in ammonium-grown cells and released by nitrate. A great diversity of molecular size of the NR-CRM of ammonium-grown cells was observed which was interpreted by the authors to indicate that the NR precursor is subject to extensive degradation within the cell. L.P.Solomonson has recently employed immunocytochemical methods to locate NR in *Chlorella* cells (personal communication). He found NR in nitrate-induced cells to be associated with the plasmalemma while in ammonium-grown cells it was randomly distributed. I would like to suggest that this altered distribution of NR is a reflection of the breakdown of NR in ammonium-grown cells. As previously noted, the process by which inactive *Chlorella* NR precursor is converted to active NR is envisaged to be

dependent on another protein. I would like to propose that this other protein may be a proteinase inhibitor whose synthesis is 'induced' by nitrate which acts to protect NR from degradation.

The observations regarding presence of NR-CRM in ammonium-grown cells of *Chlorella* are in direct contrast to those made by Amy and Garrett (1979) in ammonium-grown mycelia of *Neurospora* who showed them to contain no NR-CRM using similar immunological techniques. It is likely that the control mechanism for NR synthesis in *Chlorella* and *Neurospora* are fundamentally different while the situation in higher plants remains equivocal.

Somers *et al* (1983) also investigated the response of NR-CRM and NR activity in leaves of barley when seedlings were transferred to nitrate-free solutions or from light to dark. They concluded that the decline in NR activity and CRM was most likely to be due to protein degradation. The decrease in barley shoot NR-CRM was, however, observed to be slightly slower than the loss of NR activity when the nitrate source was removed. As higher plant NR has been shown to be susceptible to limited proteolysis (Chapter 5, Fig.32;Batt and Wallace, 1983) this suggests that some NR-CRM exists in a transient, inactive state (domains and domain-associations?) before complete degradation of the enzyme. However, immunologically reactive degradative fragments of barley NR were not observed by Somers *et al* (1983) in the SDS western blots during decay of NR activity.

Loss of NR activity in *N. crassa* due to nitrate starvation or the presence of ammonium has been shown to result from repression of enzyme synthesis and protein degradation (Amy and Garrett, 1980; Sorger *et al*, 1974) while a reversible inactivation of *Chlorella* NR leads to decrease in NR activity when induced cells are transferred to media containing ammonium (Solomonson, 1978)

Somers *et al* (1983) concluded that barley NR activity is regulated by synthesis and degradation of NR (as in *N. crassa*) and not by reversible activation and inactivation of NR (as in *Chlorella*). Interestingly, identical conclusions have very recently been drawn regarding the regulation of nitrite reductase in pea leaves (Gupta and Beevers, 1984).

Reversible inactivation of NR, particulary in response to ammonium, but also during light-dark cycles (Tischner and Hutterman, 1978) has been widely reported in algal systems. In *Chlorella* (Moreno *et al*, 1972) and *Chlamydomonas* (Herrera *et al*, 1972) it has been demonstrated that the effect of ammonium is to uncouple non-cyclic phosphorylation and the resultant increase in intracellular reducing power and ADP to directly inhibit NR activity. Inactive forms of NR have been isolated from ammonium-grown algae (Gewitz *et al*, 1978; Lorimer *et al*, 1974) and it was discovered that NR could be extracted from *C. vulgaris* in a partially inactive form even when the cells are grown in nitrate.

NADH has been shown to inactivate *C. vulgaris* NR *in vitro* whereas NR from *C. fusca* required the simultaneous presence of ADP and NADH before inactivation occurred (Maldonado *et al*, 1973). The effect of NADH on *in vitro* NR activity is not consistent. NR of rice (Leong and Shen, 1979), spinach (Palacian *et al*, 1974), wheat (Sherrard and Dalling, 1979) and maize (Wallace, 1975b) have been shown to be inactivated *in vitro* by NADH, in the absence of nitrate, while NADH has been reported to stabilise NR in sorghum (Kadam *et al*, 1980), bean (Sluiter-Scholten, 1975) cotton cotyledons (Tischner *et al*, 1978) and barley (Small, 1980). A combination of NADH and HCN, however, resulted in rapid *in vitro* inactivation of NR from all higher plant and algal sources studied. CN is thought to bind to the molybdenum of the reduced enzyme, blocking electron transfer to nitrate. The presence of HCN in extracts of *C. vulgaris* has been demonstrated by Gewitz *et al* (1974) who suggested that CN might be involved in the *in vivo* regulation of algal NR and mechanisms for the *in vivo* generation of CN have been proposed (Solomonson and Spehar, 1977; Pistorius *et al*, 1977).

Reactivation of the *in vitro* inactivated higher plant NR by FeCN (Leong and Shen, 1979; Palacian *et al*, 1974; Wallace, 1975b), light plus FAD, FMN or riboflavin (Aparicio *et al*, 1976; De La Rosa *et al*, 1976), nitrate (Palacian *et al*, 1974) and trivalent manganese ions (Maldonado *et al*, 1980; Funkhouser and Akerman, 1976) has been demonstrated. Attempts to isolate inactive forms

of higher plant NR have, however, been unsuccessful. Some evidence for the existence of an inactive form of NR *in vivo* has been provided by Arayan *et al* (1983) who showed that NR isolated during the late photoperiod and dark period from wheat plants grown with a low nitrate supply was activated by treatment with either FeCN or light. Such observations were taken to indicate the presence of an inactivated, over-reduced form of NR in these tissues which was capable of reactivation.

As reversible inactivation of higher plant NR can be readily demonstrated *in vitro* and evidence for reversibly inactivated forms of NR *in vivo* is now forthcoming it is likely that NR in higher plants may not be solely regulated by synthesis and degradation of NR as suggested by Somers *et al* (1983) but that reversible inactivation of NR may have a role to play under certain circumstances.

SECTION VII CONCLUSIONS AND THOUGHTS ON FUTURE WORK

The results presented in this thesis show clearly that barley NR is unstable in crude extracts, especially from older leaves. The problems which this instability has caused with respect to poor yields on purification of the enzyme and on interpretation of results based on estimation of NR activity in crude extracts have been illustrated. Evidence has been presented which indicates that *in vitro* instability of barley NR is due to limited proteolysis of the enzyme. The analysis of the NADH-CR species apparently released from NR by proteolytic

degradation has allowed a model to be presented for the structure of the enzyme and given insight into the genetic evolution of NR and its possible gene structure.

However much work remains to be carried out before the proposed model can be verified (or otherwise) and the significance of proteolysis determined with respect to regulation of NR activity *in vivo*.

The most obvious areas for further study include:

(1) Purify and characterise the leupeptin-sensitive proteinase envisaged to be present in barley cv Golden Promise leaves.

(2) Identify and isolate other proteinase activities in barley leaves employing the chromogenic peptide substrates described by Achstetter *et al* (1981).

(3) Raise antibodies against purified proteinases active towards NR in order to (a) determine the occurrence of the proteinases in other plants and (b) to investigate their subcellular location by immunocytological procedures.

(4) Identify and isolate any non-proteolytic or binding NR-inactivators which may be present in barley leaves.

(5) Identify and isolate any NR-stabilising factors which may be present in barley leaves.

(6) Study factors which might alter the susceptibility of NR to proteinases/inhibitors/activators e.g. presence of substrate and cofactors.

(7) Monitor the effect of environmental conditions on the levels of NR protein and the levels of factors which modulate its activity by immunological methods in order to determine how NR is regulated under these conditions.

(8) In an attempt to resolve apparently contradictory observations *re* structure of NR in barley cv Steptoe and barley cv Golden Promise (see Discussion, Section IV) - isolate NR from different barley cultivars, determine any differential sensitivity to purified preparations of NR-inactivators and compare the products of limited proteolysis.

(9) Purify and characterise NR domains released by action of trypsin and NR-active proteinases, including components derived from NR which may not possess enzyme activity (such as the proposed MoCo-binding domain).

(10) Extract and clone the gene for barley NR- determination of the base sequence of the gene would allow the complete structure of NR to be elucidated, including any domains.

BIBLIOGRAPHY

Achstetter, T., Ehmann, C. and Wolf, D.H. (1981) Arch. Biochem. Biophys. 207, 445.

Amy, N.K. and Garrett, R.H. (1979) Anal. Biochem. 95, 97.

Amy, N.K. and Garrett, R.H. (1980) J. Bacteriol. 144, 232.

Amy, N.K. and Rajagopalan, K.V. (1979) J. Bacteriol. 140, 114.

Antoine, A.D. (1974) Biochemistry 13, 2289.

Aparicio, P.J., Roldan, J.M. and Calero, F. (1976) Biochem. Biophys. Res. Commun. 70, 1071.

Arnon, R. (1973) In "The Antigens" Vol I ed. M. Sela pp 97-159 Academic Press, New York.

Arst, H.N., MacDonald, D.W. and Cove, D.J. (1970) Mol. Gen. Genet. 108, 129.

Aryan, A.P., Batt, R.G. and Wallace, W. (1983) Plant Physiol. 71, 582.

Aslam, M. (1977) Plant Sci. Lett. 9, 89.

Aslam, M. and Oaks, A. (1976) Plant Physiol. 57, 572.

Austin, R.B., Rossi, L. and Blackwell, R.D. (1978) Ann. Bot. 42, 429.

Aviram, E. (1975) Isr. Chem. Soc. 43rd. Ann. Meeting Proc. p 100.

Ballard, F.J. (1977) Essays in Biochemistry 13, 1.

Bartholomes, P., Boker, H. and Jaenicke, R. (1979) Eur. J. Biochem. 102, 167.

Batt, R.G. and Wallace, W. (1983) Biochim. Biophys. Acta 744, 205.

Beale, S.I. and Castelfranco, P.A. (1974) Plant Physiol. 53, 291.

Beers, R.F. and Sizer, I.W. (1952) J. Biol. Chem. 195, 133.

Beevers, L. and Hageman, R.H. (1969) Ann. Rev. Plant Physiol. 20, 495.

Beevers, L. and Hageman, R.H. (1972) Photophysiology 7, 85.

Beevers, L., Schrader, L.E., Flesher, D. and Hageman, R.H. (1965) Plant Physiol. 40, 691.

Bowerman, A. and Goodman, P.J. (1971) Ann. Bot. 35, 353.

Bradford, M.M. (1976) Anal. Biochem. 72, 248.

Bright, S.W.J., Norbury, P.B., Franklin, J., Kirk, D.W. and Wray, J.L. (1983) Mol. Gen. Genet. 189, 240.

Buchanan, R.J. and Wray, J.L. (1982) Mol. Gen. Genet. 189, 240.

Calvo, J.M. and Fink, G.R. (1971) Ann. Rev. Biochem. 40, 943.

Campbell, J.McA., Small, I.S. and Wray, J.L. (1984) Phytochemistry 23, 1391.

Campbell, J.McA. and Wray, J.L. (1983) Phytochemistry 22, 2375.

Campbell, W.H. (1976) Plant Sci. Lett. 7, 239.

Campbell, W.H. (1978) Z. Pflanzenphysiol. 88, 357.

Campbell, W.H. (1982) Proceedings of the International Symposium on Nitrate Assimilation - Molecular and Genetic Aspects, Gaterslaben, G.D.R. L7.

Campbell, W.H. and Smarrelli, J. (1976) Plant Physiol. 57, 637.

Campbell, W.H. and Smarrelli, J. (1978) Plant Physiol. 61, 611.

Castelfranco, P.A. and Jones, O.G.T. (1975) Plant Physiol. 55, 485.

Charney, J. and Tomarelli, R.M. (1947) J. Biol. Chem. 171, 501.

Chroboczek-Kelker, H. (1969) Ph.D. Thesis, Michigan State University.

Coddington, A. (1976) Mol. Gen. Genet. 145, 195.

Cove, D.J. (1979) Biol. Rev. 54, 291.

Crawford, I.P. (1975) Bacteriol. Rev. 39, 87.

Creswell, C.F., Hageman, R.H., Hewitt, E.J. and Hucklesby, D.P. (1965) Biochem. J. 94, 40.

Crick, F. (1979) Science 204, 264.

Dailey, F.A., Kuo, T. and Warner, R.L. (1982a) Plant Physiol. 69, 1196.

Dailey, F.A., Warner, R.L., Somers, D.A. and Kleinhofs, A. (1982b) Plant Physiol. 69, 1200.

Dalling, M.J., Boland, G. and Wilson, J.H. (1976) Aust. J. Plant Physiol. 3, 721.

Dalling, M.J. and Loyn, R.H. (1977) Aust. J. Agric. Res. 28, 1.

Dalling, M.J., Tolbert, N.E. and Hageman, R.H. (1972) Biochim. Biophys. Acta 283, 505.

Deckard, E.L., Lambert, R.J. and Hageman, R.H. (1973) Crop. Sci. 13, 343.

De La Rosa, F.F., Castillo, F., Mendez, J.M. and Palacian, E. (1976) FEBS Lett. 64, 293.

De La Rosa, M.A., Diez, J., Vega, J.M. and Losada, M. (1980) Eur. J. Biochem. 106, 249.

De La Rosa, F.F. and Palacian, E. (1981) Plant Sci. Lett. 21, 1.

De Luca, C., Weber, M.M. and Kaplar, N.O. (1956) J. Biol. Chem. 223, 559.

De Simone, J., Kleve, L., Longley, M.A. and Schaeffer, J. (1974) Biochem. Biophys. Ress. Commun. 57, 248.

Dice, J.F. and Goldberg, A.L. (1976) Nature 262, 514.

Diez, J., Chaparro, A., Vega, J.M. and Relimpio, A.M. (1977) Planta 137, 231.

Downey, R.J. (1971) J. Bacteriol. 105, 759.

Downey, R.J. (1973a) Biochem. Biophys. Res. Commun. 50, 920.

Downey, R.J. (1973b) Microbios. 7, 53.

Downey, R.J. and Focht, W.J. (1974) Microbios. 11, 61.

Downey, R.J. and Steiner, F.X. (1979) J. Bacteriol. 137, 105.

Drenth, J., Jansonius, J.N., Koekoek, R., Swen, J.M. and Wolthers, B.J. (1968) Nature 218, 929.

Duke, S.H., Freidrich, J.W., Schrader, L.E. and Koukkan, W.L. (1978) Physiol. Plant. 42, 269.

Eaglesham, A.R.J. and Hewitt, E.J. (1975) Plant Cell Physiol. 16, 1137.

Eck, H.V. and Hageman, R.H. (1974) Crop Sci. 14, 283.

Eilrich, G.L. and Hageman, R.H. (1973) Crop Sci. 13, 59.

Evans, H.J. and Hall, A. (1953) Plant Physiol. 28, 233.

Feenstra, W.J. and Jacobsen, E. (1980) Theor. Appl. Genet. 58, 39.

Fellipe, G.M., Dale, J.E. and Marriott, C. (1975) Ann. Bot. 39, 43.

Fernandez, E. and Cardenas, J. (1981) Biochim. Biophys. Acta 657, 1.

Fernandez, E. and Cardenas, J. (1982) Biochim. Biophys. Acta. 681, 530.

Fernandez, E. and Cardenas, J. (1983a) Z. Naturforsch. 38, 35.

Fernandez, E. and Cardenas, J. (1983b) Biochim. Biophys. Acta. 745, 12.

Fernandez, E. and Cardenas, J. (1983c) Z. Naturforsch. 38, 439.

Ferrari, T.E. and Varner, J.E. (1970) Proc. Natl. Acad. Sci. (Wash) 65, 729.

Fido, R.J., Hewitt, E.J., Notton, B.A., Jones, O.T.G. and Nasrulhaq-Boyce, A. (1979) FEBS Lett. 99, 180.

Filner, P. (1966) Biochim. Biophys. Acta 118, 299.

Franco, A.R., Cardenas, J. and Fernandez, E. (1984) EMBO J. 3, 1403.

Funkhouser, E.A. and Akerman, R. (1976) Eur. J. Biochem. 66, 225.

Funkhouser, E.A. and Ramadoss, C. S. (1980) Plant Physiol. 65, 944.

Garrett, R.H. and Amy, N.K. (1978) Adv. Microb. Physiol. 18, 1.

Garrett, R.H. and Cove, D.J. (1976) Mol. Gen. Genet. 149, 179.

Garrett, R.H. and Nason, A. (1967) Proc. Natl. Acad. Sci. U.S. 58, 1603.

Garrett, R.H. and Nason, A. (1969) J. Biol. Chem. 244, 2870.

Gervais, M. and Tegoni, M. (1980) Eur. J. Biochem. 111, 357.

Gewitz, H.S., Lorimer, G.H., Solomonson, L.P. and Vennesland, B. (1974) Nature 249, 79.

Gewitz, H.S., Pietke, J. and Vennesland, B. (1978) Planta 141, 323.

Giri, L. and Ramadoss, C.S. (1979) J. Biol. Chem. 254, 11703.

Gore, N.R. and Wray, J.L. (1978) Plant Physiol. 61, 20.

Graf, L., Notton, B.A. and Hewitt, E.J. (1975) Phytochemistry 14, 1241.

Granick, S., Sinclair, P., Sassa, S. and Grieninger, G. (1975) J. Biol. Chem. 250, 9215.

Guerrero, M.G. and Guitierrez, M. (1977) Biochim. Biophys. Acta 482, 272.

Guerrero, M.G., Jetschman, K. and Volker, W. (1977) Biochim. Biophys. Acta 482, 19.

Guiard, B. and Lederer, F. (1977) Eur. J. Biochem. 74, 181.

Gupta, S.C. and Beevers, L. (1984) Plant Physiol. 75, 251.

Gustafson, G. and Ryan, C.A. (1976) J. Biol. Chem. 251, 7004.

Hageman, R.H. (1979) In "Nitrogen Assimilation of Plants" eds. Hewitt, E.J. and Cutting, C.V. pp 591-612, Academic Press.

Hageman, R.H. and Flesher, D. (1960) Plant Physiol. 35, 700.

Hageman, R.H. and Hucklesby, D.R. (1971) Methods Enzymol. 23, 491.

Hageman, R.H., Lambert, R.J., Loussart, D., Dalling, M. and Klepper, L.A. (1976) In "Genetic Improvement of Seed Proteins" p 103, Natl. Acad. Sci. U.S.A., Washington, D.C.

Hageman, R.H., Leng, E.R. and Dudley, J.W. (1967) Adv. Agron. 19, 45.

Hageman, R.H., Zieserl, J.F. and Leng, E.R. (1963) Nature 197, 263.

Hale, G. and Perham, N. (1979) Eur. J. Biochem. 94, 119.

Hamano, T., Oji, Y., Okamoto, S., Mitsuhashi, Y. and Matsuki, Y. (1983) Plant Cell Physiol. 24, 1337.

Hamano, T., Oji, Y., Okamoto, S., Mitsuhashi, Y. and Matsuki, Y. (1984) Plant Cell Physiol. 25, 419.

Harper, J.E. and Hageman, R.H. (1972) Plant Physiol. 49, 146.

Haynes, R.J. and Goh, K.M. (1978) Biological Reviews 53, 465.

Heimer, Y.M., Krashin, S. and Riklis, E. (1976) FEBS Lett. 62, 30.

Heimer, Y.M., Wray, J.L. and Filner, P. (1969) Plant Physiol. 44, 1197.

Herrera, J., Paneque, A., Maldonado, J.M., Barea, J.L. and Losada, M. (1972) Biochem. Biophys. Res. Commun. 48, 996.

Herrero, A., De La Rosa, M.A., Diez, J. and Vega, J.M. (1980) Plant Sci. Lett. 17, 409.

Hewitt, E.J. (1951) Ann. Rev. Plant Physiol. 2, 25.

Hewitt, E.J. (1975) Ann. Rev. Plant Physiol. 26, 73.

Hewitt, E.J., Hucklesby, D.P. and Notton, B.A. (1976) In "Plant Biochemistry" eds. D. Bonner and J.E. Varner, 3rd Edition, pp 633-681, Academic Press.

Hewitt, E.J., Notton, B.A. and Garner, C.D. (1979) Biochem. Soc. Trans. 7, 629.

Hipkin, C.R. and Syrett, P.J. (1977) J. Exp. Bot. 28, 1270.

Hoagland, P.R. and Arnon, D.I. (1938) Circ. Calif. Agric. Exp. Sta. no. 347.

Horner, R.D. (1983) Biochim. Biophys. Acta 744, 7.

Howard, W.D. and Solomonson, L.P. (1978) Proc. 176th Natl. Am. Chem. Soc. Meeting. Abstr. 89.

Howard, W.D. and Solomonson, L.P. (1981) J. Biol. Chem. 256, 12725.

Howard, W.D. and Solomonson, L.P. (1982) J. Biol. Chem. 257, 10243.

Hucklesby, D.P. and Hageman, R.H. (1973) Anal. Biochem. 56, 591.

Ingle, J., Joy, K.W. and Hageman, R.H. (1966) Biochem. J. 100, 577.

Jackson, W.A., Flesher, D. and Hageman, R.H. (1973) Plant Physiol. 51, 120.

Jacq, C. and Lederer, F. (1974) Eur. J. Biochem. 41, 311.

Jaworski, E.G. (1971) Biochem. Biophys. Res. Commun. 43, 1274.

Johnson, J.L. (1980) In "Molybdenum and Molybdenum-containing Enzymes", ed. M. Coughlan, pp 345-383, Pergamon Press.

Johnson, J.L., Hainline, B.E. and Rajagopalan, K.V. (1980) J. Biol. Chem. 255, 1783.

Johnson, J.L., Jones, H.P. and Rajagopalan, K.V. (1977) J. Biol. Chem. 252, 4994.

Johnson, J.L. and Rajagopalan, K.V. (1977) J. Biol. Chem. 252, 2017.

Jolly, S.O., Campbell, W.H. and Tolbert, N.E. (1976) Arch. Biochem. Biophys. 174, 431.

Jolly, S.O. and Tolbert, N.E. (1978) Plant Physiol. 62, 197.

Jones, R.W., Abbot, A.J., Hewitt, E.J., James, D.M. and Best, G.R. (1977) Planta 133, 27.

Kadam, S.S., Gandhi, A.P., Sawhney, S.K. and Naik, M.S. (1975) Ind. J. Biochem. Biophys. 12, 81.

Kadam, S.S., Johari, R.P., Ramaro, C.S. and Srinivasan, (1980) Phytochemistry 19, 2095.

Kaplan, D., Mayer, A.M. and Lips, S.H. (1978) Planta 138, 205.

Katanuma, N., Kominami, E., Banno, Y., Kito, K., Aoki, Y. and Urata, G. (1976) In "Advances in Enzyme Regulation" Vol 14 ed. G. Weber pp 325-346, Pergamon Press.

Ketchum, P.A. and Downey, R.J. (1975) Biochim. Biophys. Acta 385, 354.

King, J. and Khanna, V. (1980) Plant Physiol. 66, 632.

Kirchner, K. and Bisswanger, H. (1976) Ann. Rev. Biochem. 45, 143.

Kleinhofs, A., Kuo, T.M. and Warner, R.L. (1980) Mol. Gen. Genet. 177, 421.

Kleinhofs, A., Taylor, J., Kuo, T.M., Somers, D.A. and Warner, R.L. (1983) In "Genetic Engineering in Eukaryotes" Eds. P.F. Lurquin and A. Kleinhofs, pp 215-231, Plenum Publishing Corporation.

Kleinhofs, A., Warner, R.L., Meulbauer, F.J. and Nilan, R.A. (1978) Mutation Res. 51, 29.

Knypl, J.S. (1974) In "Mechanisms of Regulation of Plant Growth" eds. R.L. Bieleski, A.R. Ferguson and M.M. Creswell, p 71, Bull. Roy. Soc. New Zealand.

Kresze, G.B. and Ronft, H. (1981) Eur. J. Biochem. 119, 573.

Kuo, T. (1979) Ph.D. Dissertation, Washington State University, Pullman.

Kuo, T., Kleinhofs, A., Somers, D. and Warner, R. (1981) Mol. Gen. Genet. 181, 20.

Kuo, T. , Kleinhofs, A. and Warner, R.L. (1980) Plant Sci. Lett. 17, 371.

Kuo, T. , Somers, D.A., Kleinhofs, A. and Warner, R.L. (1982b) Biochim. Biophys. Acta 708, 75.

Kuo, T. , Warner, R.L. and Kleinhofs, A. (1982a) Phytochemistry 21, 531.

Labeyrie, F., Groudinsky, O., Jacquot-Armand, V. and Naslin, L. (1966) Biochim. Biophys. Acta 128, 492.

Laemmli, U.K. (1970) Nature 227, 680.

Lam, T.H. and Shaw, M. (1970) Biochem. Biophys. Res. Commun. 39, 965.

Laskowski, M. and Kato, I. (1980) Ann. Rev. Biochem. 49, 593.

Le, K.H. and Lederer, F. (1983) EMBO J. 2, 1909.

Lee, K-Y., Pan, S-S., Erickson, R. and Nason, A. (1974) J. Biol. Chem. 249, 3941.

Leong, C.C. and Shen, T.C. (1979) Experentia 35, 584.

Leong, C.C. and Shen, T.C. (1982) Biochim. Biophys. Acta 703, 129.

Lewin, R. (1979) New Scientist 82, 452.

Lewis, N.J. (1975) Ph.D. Thesis, University of Cambridge.

Lewis, O.A.M., James, D.M. and Hewitt, E.J. (1982) Ann. Bot. 49, 39.

Lin, S. and Zabin, I. (1972) J. Biol. Chem. 247, 2205.

Loomis, W.D. (1974) Methods Enzymol. 31, 528.

Lorimer, G.H., Gewitz, H.S., Volker, W., Solomonson, L.P. and Vennesland, B. (1974) J. Biol. Chem. 249, 6074.

MacDonald, D.W. and Coddington, A. (1974) Eur. J. Biochem. 46, 169.

MacDonald, D.W. and Cove, D.J. (1974) Eur. J. Biochem. 47, 107.

MacDonald, D.W., Cove, D.J. and Coddington, A. (1974) Mol. Gen. Genet. 128, 187.

MacKinney, G. (1941) J. Biol. Chem. 140, 315.

Maldonado, J.M., Herrera, J., Paneque, A. and Losada, M. (1973) Biochem. Biophys. Res. Commun. 51, 27.

Maldonado, J.M., Notton, B.A. and Hewitt, E.J. (1978) Plant Sci. Lett. 13, 143.

Maldonado, J.M., Notton, B.A. and Hewitt, E.H. (1980) Planta 150, 242.

Mayer, R.J. and Walker, J.H. (1978) In "Techniques in Protein and Enzyme Biochemistry", B119: pp 1-32, Elsevier/North Holland, Biomedical Press.

Mechler, B. and Wolf, D.H. (1981) Eur. J. Biochem. 121, 47.

Mendel, R.R. (1980) Biochem. Physiol. Pflanzen. 175, 216.

Mendel, R.R., Alikulov, Z.A., Lvov, N.P. and Muller, A.J. (1981) Mol. Gen. Genet. 181, 395.

Mendel, R.R., Alikulov, Z.A. and Muller, A.J. (1982) Plant Sci. Lett. 25, 67.

Mendel, R.R. and Muller, A.J. (1979) Mol. Gen. Genet. 177, 145.

Mendel, R.R. and Muller, A.J. (1980) Plant Sci. Lett. 18, 277.

Mevel-Ninio, M. (1972) Eur. J. Biochem. 25, 254.

Miflin, B.J. and Lea, P.J. (1976) Phytochemistry 15, 873.

Miflin, B.J. and Lea, P.J. (1977) Ann. Rev. Plant Physiol. 28, 299.

Mikola, J. and Kolehmainen, L. (1972) Planta 104, 167.

Miller, B. and Huffaker, R. (1980a) 65, S179.

Miller, B. and Huffaker, R. (1980b) 65, S180.

Miller, B.L. and Huffaker, R.C. (1981) Plant Physiol. 68, 930.

Miller, B.L. and Huffaker, R.C. (1982) Plant Physiol. 69, 58.

Minagawa, N. and Yoshimoto, A. (1982) J. Biochem. 91, 761.

Moreno, C.G., Aparicio, P.J., Palacian, E. and Losada, M. (1972) FEBS Lett. 26, 11.

Muller, A.J. (1982) Proceedings of the International Symposium on Nitrate Assimilation - Molecular and Genetic Aspects, Gaterslaben, GDR. L8.

Muller, A.J. and Grafe , R. (1978) Mol. Gen. Genet. 161, 67.

Naik, M.S., Abrol, Y.P., Nair, T.V.R. and Ramaro, C.S. (1982) Phytochemistry 21, 495.

Nandi, D.L. and Shemin, D. (1968) J. Biol. Chem. 243, 1236.

Narayanan, K.R., Somers, D.A., Kleinhofs, A. and Warner, R.L. (1983) Mol. Gen. Genet. 190, 222.

Naslin, L., Spyridakis, A. and Labeyrie, F. (1973) Eur. J. Biochem. 34, 268.

Nason, A., Antoine, A.D., Ketchum, P. A., Frazier, W.A. and Lee, D.K. (1970) Proc. Natl. Acad. Sci. U.S. 65, 137.

Nason, A., Lee K-Y., Pan, S-S., Ketchum, P.A., Lamberti, A. and De Vries, J. (1971). Proc. Natl. Acad. Sci. U.S. 68, 3242.

Nasrulhaq-Boyce, A. and Jones, O.T.G. (1977) Planta 137, 77.

Nicholas, D.J.D. and Nason, A. (1954a) Arch. Biochem. Biophys. 51, 311.

Nicholas, D.J.D. and Nason, A. (1954b) J. Biol. Chem. 207, 353.

Nicholas, D.J.D. and Nason, A. (1954c) J. Biol. Chem. 211, 183.

Notton, B.A., Fido, R.J. and Hewitt, E.J. (1977) Plant Sci. Lett. 8, 165.

Notton, B.A., Fido, R.J. and Hewitt, E.J. (1979) Abstract of the XIth IUB Conference, Toronto, Abstract 03-2-S175.

Notton, B.A. and Hewitt, E.J. (1971a) Plant Cell Physiol. 12, 465.

Notton, B.A. and Hewitt, E.J. (1971b) FEBS Lett. 18, 19.

Notton, B.A. and Hewitt, E.J. (1971c) Biochem. Biophys. Res. Commun. 44, 702.

Notton, B.A. and Hewitt, E.J. (1979) In "Nitrogen Assimilation of Plants" eds. E.J. Hewitt and C.V. Cutting, pp 227-244, Academic Press.

Notton, B.A., Hewitt, E.J. and Fielding, A.H. (1972) Phytochemistry 11, 2447.

Notton, B.A., Icke, D. and Hewitt, E.J. (1976) Rep. Long Ashton Res. Station for 1975, 63.

Oaks, A. (1974) Biochim. Biophys. Acta 372, 112.

Oaks, A. (1979) In "Nitrogen Assimilation of Plants" eds. E.J. Hewitt and C.V. Cutting, pp 217-226, Academic Press.

Oaks, A., Aslam, M. and Boesel, I. (1977) Plant Physiol. 59, 391.

Oaks, A., Wallace, W. and Stevens, D. (1972) Plant Physiol. 50, 649.

Oncley, J.L. (1941) Ann. N.Y. Acad. Sci. 41, 121.

Oostinder-Braaksma, F.J. and Feenstra, W.J. (1973) Mutation Res. 19, 175.

Orebamjo, T.O. and Stewart, G.R. (1975a) Planta 122, 27.

Orebamjo, T.O. and Stewart, G.R. (1975b) Planta 122, 37.

Orihuel-Iranzo, B. and Campbell, W.H. (1980) Plant Physiol. 65, 595.

Palacian, E., De La Rosa, F., Castillo, F. and Morena, C.G. (1974) Arch. Biochem. Biophys. 161, 441.

Pan, Y.T. and Marsh, H.V. (1972) Plant Physiol. 49, S49.

Pan, S.S. and Nason, A. (1978) Biochim. Biophys. Acta 523, 297.

Paneque, A., Del Campo, F.F., Ramirez, J.M. and Losada, M. (1965) Biochim. Biophys. Acta 109, 79.

Parish, R.W. (1975a) Planta 123, 1.

Parish, R.W. (1975b) Planta 123, 15.

Parkash, V. (1972) Planta 102, 372.

Pate, J.S. (1973) Siol Biol. Biochem. 5, 109.

Pateman, J.A., Cove, D.J., Rever, B.M. and Roberts, D.B. (1964) Nature 201, 58.

Pateman, J.A., Rever, B.M. and Cove, D.J. (1967) Biochem. J. 104, 103.

Pienkos, P.T., Shah, V.K. and Brill, W.J. (1977) Proc. Natl. Acad. Sci. U.S. 74, 5468.

Pistorius, E.K., Gewitz, H.S., Voss, H. and Vennesland, B. (1977) Biochim. Biophys. Acta 481, 384.

Platt, T., Miller, J. and Weber, K. (1970) Nature 228, 1154.

Porath, J. (1963) Pure Appl. Chem. 6, 233.

Poule, M. and Oaks, A. (1983) Plant Physiol. 72, S78.

Premakumar, K., Sorger, G.J. and Gooden, D. (1978) Biochim. Biophys. Acta 519, 275.

Purvis, A.C., Tischler, C.R. and Fites, R.C. (1976) Plant Physiol. 58, 95.

Purvis, A.C., Tischler, C.R. and Funkhouser, E.A. (1980) Physiol. Plant 48, 389.

Radin, J.W. (1974) Plant Physiol. 53, 458.

Radin, J.W. (1975) Plant Physiol. 55, 178.

Radin, J.W. (1977) Aus, J. Plant Physiol. 4, 811.

Ragster, L. and Chrispeels, M.J. (1979) Plant Physiol. 64, 857.

Rajagopalan, K.V., Johnson, J.L. and Hainline, B.E. (1980) Fed. Proc. 40, 1652.

Redinbaugh, M.G. and Campbell, W.H. (1983) Plant Physiol. 71, 205.

Reed, A.J., Below, F.E. and Hageman, R.H. (1980) Plant Physiol. 66, 164.

Reisenauer, H.M. (1978) In "Nitrogen in the Environment" Vol II, eds. D.R. Nielsen and J.G. MacDonald, pp 157-170, Academic Press.

Relimpio, A.M., Aparicio, P.J., Paneque, A. and Losada, M. (1971) FEBS Lett. 17, 226.

Renosto, F., Schmidt, N.D. and Segel, I.H. (1982) Proceeding of the International Symposium of Nitrate-Assimilation - Molecular and Genetic Aspects, Gaterslaben, GDR. L14.

Reynolds, T. (1977) Ann. Bot. 41, 637.

Ritenour, G.L., Joy, K.W., Bunning, J. and Hageman, R.H. (1967) Plant Physiol. 42, 233.

Roldan, J.M., Verbelen, J-P., Butler, W.L. and Tokuyasa, K. (1982) Plant Physiol. 70, 872.

Roustan, J-L., Neuberger, M. and Fourcy, A. (1974) Physiol. Veg. 12, 527.

Rucklidge, G.J., Notton, B.A. and Hewitt, E.J. (1976) Biochem. Soc. Trans. 4, 77.

Ryan, C.A. and Walker-Simmons, M. (1981) In "The Biochemistry of Plants", Vol 6 Proteins and Nucleic Acids, ed. A. Marcus, pp 321-350, Academic Press.

Sanderson, G.W. and Cocking, E.C. (1964) Plant Physiol. 39, 416.

Satler, S.O. and Tfumann, K.V. (1980) Plant Physiol. 66, 395.

Schimke, R.T. (1975) In "Intracellular Protein Turnover" eds. R.T. Schimke and R.T. Katunuma, pp 173-186, Academic Press.

Schader, L.E., Beevers, L. and Hageman, R.H. (1967) Biochem. Biophys. Res. Commun. 26, 14.

Schrader, L.E., Cataldo, D.A. and Peterson, D.M. (1974a) Plant Physiol. 53, 688.

Schrader, L.E., Cataldo, D.A., Peterson, D.M. and Vogelzang, R.D. (1974b) Physiol. Plant. 32, 337.

Schrader, L.E., Domska, D., Jung, P.E. and Peterson, L.A. (1972) Agron. J. 64, 690.

Schrader, L.E., Ritenour, G.L., Eilrich, G.L. and Hageman, R.H. (1968) Plant Physiol. 43, 930.

Segal, H.L. (1976) Current Topics in Cell. Reg. 11, 183.

Shah, V.K. and Brill, W.J. (1977) Proc. Natl. Acad. Sci. U.S. 74, 3249.

Shannon, J.D. and Wallace, W. (1979) Eur. J. Biochem. 102, 399.

Shen, T-C. (1976) Planta 108, 21.

Shen, T-C., Funkhouser, E. A. and Guerrero, M.G. (1976) Plant Physiol. 58, 292.

Sherrard, J.H. and Dalling, M.J. (1978) Ann. Bot. 42, 1421.

Sherrard, J.H. and Dalling, M.J. (1979) Plant Physiol. 63, 346.

Sherrard, J.H., Kennedy, J.A. and Dalling, M.J. (1979a) Plant Physiol. 64, 439.

Sherrard, J.H., Kennedy, J.A. and Dalling, M.J. (1979b) Plant Physiol. 64, 640.

Siegel, L.M. and Monty, K.J. (1966) Biochim. Biophys. Acta 112, 346.

Siegel, N.R. and Enns, R.K. (1979) Plant Physiol. 63, 206.

Sihag, R.K., Guha-Mukherjee, S. and Sopory, S.K. (1979) Plant Physiol. 45, 281.

Sluiters-Scholten, C.M.T.H. (1975) Planta 123, 175.

Small, I.S. (1980) Ph.D. Thesis, St. Andrews University.

Small, I.S. and Wray, J.L. (1980a) Phytochemistry 19, 387.

Small, I.S. and Wray, J.L. (1980b) Plant Sci. Lett. 18, 389.

Smarrelli, J. and Campbell, W.H. (1979) Plant Sci. Lett. 16, 139.

Smarrelli, J. and Campbell, W.H. (1981) Plant Physiol. 68, 1226.

Smarrelli, J. and Campbell, W.H. (1982) Proceedings of the International Symposium on Nitrate Assimilation - Molecular and Genetic Aspects, Gaterslaben, G.D.R. P27.

Smith, E.L. (1970) The Enzymes 1, 267.

Smith, S.C. (1983) M.Sc. Thesis, University of St. Andrews.

Smith, F.W. and Thompson, J.F. (1971) Plant Physiol. 48, 219.

Snell, F.D. and Snell, C.T. (1949) In "Colourimetric Methods of Analysis II", 3rd Edition, ed. Van Nostrand, New York.

Solomonson, L.P. (1975) Plant Physiol. 56, 853.

Solomonson, L.P. (1978) In "Microbiology" ed. D. Schlessinger pp 315-319, American Society of Microbiology.

Solomonson, L.P., Lorimer, G.H., Hall, R.L., Borchers, R. and Bailey, J.L. (1975) J. Biol. Chem. 250, 4120.

Solomonson, L.P. and Spehar, A.M. (1977) Nature 265, 373.

Solomonson, L.P. and Vennesland, B. (1972) Biochim. Biophys. Acta 267, 544.

Somers, D.A., Kuo, T. , Kleinhofs, A. and Warner, R.L. (1982) Plant Sci. Lett. 24, 261.

Somers, D.A., Kuo, T., Kleinhofs, A. and Warner, R.L. (1983a) Plant Physiol. 71, 145.

Somers, D.A., Kuo, T. , Kleinhofs, A., Warner, R.L. and Oaks, A. (1983b) Plant Physiol. 72, 949.

Sorger, G.J. (1966) Biochim. Biophys. Acta 118, 484.

Sorger, G.J., Debanne, M.T. and Davies, J. (1974) Biochem. J. 140, 395.

Sorger, G.J., Premakumar, R. and Gooden, D. (1978) Biochim. Biophys. Acta 540, 33.

Sosa, F.H., Orkega, T. and Barea, J.L. (1978) Plant Sci. Lett. 11, 51.

Southerland, W.H. and Rajagopalan, K. V. (1978) J. Biol. Chem. 253, 8753.

Steinberg, R.A. (1937) J. Agr. Res. 57, 569.

Stewart, G.R. (1972) J. Exp. Bot. 23, 171.

Stewart, G.R. and Rhodes, D. (1977) In "Regulation of Enzyme Synthesis and Activity in Higher Plants" ed. H. Smith, pp 1-19, Academic Press.

Stravistava, H.S. (1980) Phytochemistry 19, 725.

Streit, L. and Feller, E. (1982) Experentia 38, 1176.

Tachiki, T. and Nason, A. (1983) Biochim. Biophys. Acta 744, 16.

Thayer, S.S. and Huffaker, R.C. (1984) Plant Physiol. 75, 70.

Thomas, P. E., Ryan, D. and Levin, W. (1976) Anal. Biochem. 75, 168.

Thomas, S.T., Cass, K.H. and Stellwagen, E. (1975) Proc. Natl. Acad. Sci. U.S. 72, 669.

Tischler, C.R., Purvis, A.C. and Jordan, W.R. (1978) Plant Physiol. 61, 714.

Tischner, R. A. and Huttermann, A. (1978) Plant Physiol. 62, 284.

Tokarev, B.I. and Shumney, V.K. (1977) Genetika, Moskva 13, 2097.

Travis, R.L., Huffaker, R.C. and Key, J.L. (1970a) Plant Physiol. 46, 800.

Travis, R.L., Jordan, W.R. and Huffaker, R.C. (1969) Plant Physiol. 44, 1150.

Travis, R.L., Jordan, W. R. and Huffaker, R.C. (1970b) Physiol. Plant. 23, 678.

Travis, R.L. and Key, J.L. (1971) Plant Physiol. 48, 617.

Truffa-Bachi, P. and Cohen, G.N. (1973) Ann. Rev. Biochem. 42, 113.

Upcroft, J.A. and Done, J. (1972) Plant Physiol. 42, 280.

Upcroft, J.A. and Done, J. (1976) Aust. J. Plant Physiol. 3, 421.

Vallee, B.L. and Hoch, F.L. (1955) Proc. Natl. Acad. Sci. U.S. 41, 327.

Vega, J.M. and Kamin, H. (1977) J. Biol. Chem. 252, 896.

Wallace, W. (1973a) Plant Physiol. 52, 191.

Wallace, W. (1973b) Plant Physiol. 52, 197.

Wallace, W. (1974) Biochim. Biophys. Acta 341, 265.

Wallace, W. (1975a) Plant Physiol. 55, 774.

Wallace, W. (1975b) Biochim. Biophys. Acta 377, 239.

Wallace, W. (1978) Biochim. Biophys. Acta 524, 418.

Wallace, W. and Johnson, C.B. (1978) Plant Physiol. 61, 748.

Wallace, W. and Shannon, J.D. (1981) Aust. J. Plant Physiol. 8, 211.

Walls, S., Sorger, G.J., Gordon, D. and Klein, V. (1978) Biochim. Biophys. Acta 540, 24.

Warner, R.L., Kleinhofs, A. and Muehlbauer, F.J. (1982) Crop. Sci. 22, 289.

Warner, R.L., Lin, C.J.and Kleinhofs, A. (1977) Nature 269, 406.

Wells, G.N. and Hageman, R.H. (1974) Plant Physiol. 54, 136.

White, T.J., Ibrahami, I.M. and Wilson, A.C. (1978) Nature 274, 92.

Wolf, D.H. (1982) Trends Biochem. Sci. 1, 35.

Wray, J.L. and Filner, P. (1970) Biochem. J. 119, 715.

Wray, J.L. and Kirk, D.W. (1981) Plant Sci. Lett. 23, 207.

Wray, J.L., Small, I.S. and Brown, J. (1979) Biochem. Soc. Trans. 1, 741.

Yamamoto, Y. (1963) Plant Physiol. 38, 45.

Yamaya, T. and Ohira, K. (1976) Plant Cell Physiol. 17, 633.

Yamaya, T. and Ohira, K. (1977) Plant Cell Physiol. 18, 915.

Yamaya, T. and Ohira, K. (1978a) Plant Cell Physiol. 19, 211.

Yamaya, T. and Ohira, K. (1978b) Plant Cell Physiol. 19, 1085.

Yamaya, T., Oaks, A. and Boesel, I.L. (1980a) Plant Physiol. 65, 141.

Yamaya, T., Solomonson, L.P. and Oaks, A. (1980b) Plant Physiol. 65, 146.

Zalkin, H. (1973) Adv. Enzymol. 38, 1.

Zielke, H.R. and Filner, P. (1971) J. Biol. Chem. 246, 1772.

Zumft, N.G., Aparicio, P.J., Paneque, A. and Losada, M. (1970) FEBS Lett. 9, 157.